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BIOCHEMICAL FACTORS IN THE MAXIMAL GROWTH OF TETRAHYMENA[1]

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Introduction

The earliest attempt to elucidate the nutritional requirements of the ciliate *Tetrahymena geleii* (*Glaucoma piriformis*) by the pure culture technique was made by Lwoff (1924). At that time the failure of the ciliate to grow in solutions of pure amino acids was attributed to a lack of specific chemical supplements. Later Lwoff (1932) suggested that a requirement for polypeptides was responsible for the lack of growth in such media.

This early work indicates immediately that the problem of the nutrition of *T. geleii* is a dual one. No investigation of the nitrogen requirements may be made without some knowledge of the supplementary factors needed. So far it has been shown that thiamine is important if not absolutely necessary to the nutrition of *T. geleii,* while riboflavin, pantothenic acid, nicotinic acid and pyridoxine probably play a part (Elliott, 1935b, 1939; Lwoff and Lwoff, 1937, 1938; Hall, 1940a, b, 1942; Baker and Johnson, 1941; Kidder and Dewey, 1942). In a preliminary report Dewey (1941) indicated that other factors of unknown nature are required for maximal growth. A great deal less work has been done on the nitrogen nutrition (Elliott, 1935a; Hall and Elliott, 1935; Dewey, 1941; Hall, 1942). None of the results of these investigations is conclusive. The report of Kline (1943) that *T. geleii* (*Colpidium striatum*) will grow in an amino acid solurequires confirmation.

An attempt to obtain knowledge of the supplementary requirements necessitates the use of a basic medium capable of supplying the nitrogen and carbon needs of the organism and ideally completely lacking in supplementary factors; the testing of all known growth promoting substances, and the search for and purification of possible unknown growth factors.

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Material and Methods

Preliminary experiments were carried out using four strains of *Tetrahymena geleii* and one strain of *T. vorax*. Since only quantitative differences were found between these strains, later work was confined to strain W (Claff, 1940; Kidder, 1941a). This strain was selected because it showed the most rapid growth rate and the greatest resistance to increased salt concentration of the medium. Certain experiments were also carried out using strain H, obtained from Dr. R. H. Hall.

All experimental media were prepared with water distilled twice in an all-Pyrex still over potassium permanganate and all the glassware used was Pyrex. Glassware was cleaned by soaking for at least one hour in a hot saturated solution of trisodium phosphate or a preparation sold commercially as "Keego," followed by careful rinsing to remove all traces of the cleaning agent.

Stock cultures were maintained in a 2 per cent solution of Difco proteose-peptone and also for a time in a one per cent solution of crude casein (Eimer and Amend). The fact that these ciliates could be maintained in solutions of crude casein suggested the use of a highly purified casein as a basic medium. Casein Harris (highest chemical purity) was used as a one per cent solution. Although this preparation has been called "vitamin-free" casein, this term is somewhat misleading in that traces of certain growth substances appear to be present in amounts sufficient to affect the growth of protozoa. It is, however, still adequate as a basic medium for testing responses to growth stimulants because the growth in it alone is still far from optimal with regard either to the rate of growth or the size of the population supported. For the purposes of this paper the growth in crude casein is regarded as being optimal.

Since casein, in order to be available to the ciliates, must be in solution or at least colloidal suspension, the following method was used to disperse it. To the casein was added sufficient alcohol to completely wet it, then water containing 1.0 to 1.5 ml. of normal NaOH per gram of casein was poured in slowly with stirring. With vitamin-free casein it was found necessary to add a balanced salt solution in order to obtain growth. For this purpose the modified Osterhout solution employed by Barker and Taylor (1931) was used in all but the first series of experiments. The concentrated stock solutions were added to the water used to make up the medium. The suspension of casein was allowed to stand with occasional stirring until solution was complete. Then normal HCl was added drop by drop with stirring between drops until the alkali had been neutralized. Care must be taken to add the acid slowly in order to prevent precipitation of the casein. The reaction of the medium was adjusted to pH 6.8–pH 7.0. The medium was then dispensed into tubes in amounts of 5 ml., plugged with cotton and autoclaved for 15 min. at 15 lbs. pressure. Elliott's (1939) report that vitamin-free casein required digestion with pepsin before it could be utilized by the ciliates is possibly due to a failure to put the casein into solution.

Experimental cultures were examined for growth, the results recorded and transplants made at intervals of 48 hours in casein media. The interval was slightly longer in gelatin media. The tubes were kept, however, for from one to two weeks and re-examined at intervals. Such a procedure gives results which indicate the presence or absence of factors necessary to maintain growth at or near a maximum rate. These results cannot, of course, be compared with those obtained by in-

cubating cultures for a week or more before examination, since by that time a slowly growing culture may have reached a concentration equal to that of a rapidly growing culture. In order to eliminate the effects of carry-over only the results of the third transplant in a given medium are considered. Growth is recorded as zero to four plus by comparison with growth in a control medium. Growth recorded as zero may indicate survival of the inoculum or an increase of one or two divisions, while four plus growth represents a population of from 75,000 to 100,000 organisms per ml. Two or three plus growth is intermediate. The cultures were kept at room temperature (20°–22° C.). Transplants were made using an open bacteriological loop and ordinary bacteriological technique.

Cultures were also incubated in Kidder culture flasks. The third transplant in a tube was inoculated into the flask and the growth followed by making counts at intervals of 12 or 24 hours (Kidder, 1941a).

Gelatin in concentrations of 1 per cent, 1.5 per cent and 2 per cent was also used in certain experiments. Both Harris gelatin (vitamin-free) and Eastman de-ashed gelatin were used. Another medium consisted of 1 per cent silk peptone (Seidenpepton, Hoffman-LeRoche). These media were in some cases supplemented with amino acids in various concentrations as well as with various growth promoting substances. In other cases solutions of the pure amino acids alone were used. The following amino acids were obtained from the Eastman Kodak Co.: l-histidine, l-leucine, dl-threonine, dl-β-phenylalanine, dl-methionine, d-arginine carbonate and d-lysine hydrochloride. From the Hoffman-LaRoche Co. l-tryptophane, d-isoleucine, dl-valine and glycine were obtained and from Eimer and Amend, tyrosine.

The basic medium was supplemented with vitamins and growth factors of known chemical composition as well as with crude extracts of animal and plant material. The known compounds were supplied (with one exception to be noted later) at a level of 0.001 mg. per ml. except in the case of i-inositol, which was used in a concentration of 0.004 mg. per ml., and biotin, which was used in a concentration of 0.00008 mg. per ml. Thiamine hydrochloride and riboflavin were obtained from the Hoffman-LaRoche Co. A sample of calcium pantothenate was obtained from Dr. R. J. Williams and subsequent calcium pantothenate as well as biotin methyl ester from the S. M. A. Corp. Pyridoxin hydrochloride (first used as factor I concentrate of Lepkovsky) was obtained from Merck and Co. and nicotinic acid, pimelic acid, i-inositol, uracil and p-aminobenzoic acid from the Eastman Kodak Co.

Water extracts of crude casein, egg yolk, yeast (Harris), timothy hay and alfalfa meal (Denver Milling Co.) were also used. Only the last two were used in routine culturing and in experiments on fractionation. The extracts were prepared by boiling 50 g. of material with a liter of water for ten min. and filtering with suction using Celite, analytical grade (Johns-Manville), as a filter aid. The timothy extract was used in a dilution of 1 : 5 and the alfalfa extract in a dilution of 1 : 10.

The crude extracts were treated in various ways in an attempt to remove the protein present as a preliminary to a study of the nitrogen requirements of *T. geleii*. The results of these fractionations were so interesting that studies on them were continued while the work on the nitrogenous nutrition was in progress. Tests for protein or its degradation products were made by the ninhydrin reaction.

One of the first methods tried for the removal of protein was precipitation with

lead acetate. A 25 per cent solution of normal lead acetate was added to the extract until precipitation was complete. The precipitate was then filtered off with the aid of suction and Celite. Excess lead was removed from the filtrate and the precipitate was decomposed by the use of phosphate. In the first experiments a 5 per cent solution of phosphoric acid was used but later a saturated solution of trisodium phosphate was found to give better results. The precipitated lead phosphate was then removed by filtration with suction. A similar technique was used in the preparation of the fractions obtained with ferric oxide hydrosol (prepared according to the method of Thomas and Frieden, 1923).

Barium hydroxide was used as a precipitant after the addition of three volumes of alcohol to the extract. This latter step was necessary because barium hydroxide alone caused little precipitation when added to the aqueous extract. In this case barium was removed by the use of sulfuric acid and the alcohol by boiling. When phosphotungstic acid was used the extract was first made acid by the addition of sulfuric acid to give a concentration of 50 per cent. The sulfuric acid alone caused the formation of a precipitate which was removed by filtration before the addition of the phosphotungstic acid. After separation of the phosphotungstic acid precipitate both the filtrate and the precipitate were treated with barium hydroxide to remove the sulfuric acid and the phosphotungstic acid.

The method given by Peters and Van Slyke (1931) for the removal of carbohydrate by the use of copper sulfate and calcium hydroxide was tried and found to remove all the reducing sugars present in the extracts, although protein was not removed. Excess calcium was removed from the filtrate either as the carbonate or the phosphate.

Precipitation was also carried out by the use of alcohol or acetone. After adding sufficient alcohol or acetone to give the desired concentration the extracts were allowed to stand until flocculation was complete. The precipitates were then filtered off. To remove the precipitant the filtrates were boiled or distilled, sometimes under reduced pressure. The precipitates were redissolved in water. The above named solvents as well as ether or acetic acid were also used to make extracts of alfalfa meal, using a Soxhlet apparatus except in the case of the acetic acid. The solvents were removed from the extracts by boiling and the residues taken up in water.

When it was found that the active material in these extracts was adsorbed on charcoal (Norit) and to some extent on Super Filtrol (Filtrol Corp.), attempts were made to obtain elution. Various concentrations of methyl and ethyl alcohols at various pH's were tested. The most successful eluting agent consisted of 50 per cent ethyl alcohol containing 10 per cent ammonium hydroxide. Both of these substances could be removed from the eluates by boiling.

Dialysis of the extracts was carried out in cellophane against distilled water. During the process, which lasted for several days (changing the water outside the cellophane at intervals of 12 hours), a temperature of from 50° to 60° C. was maintained in order to prevent bacterial action. An electric light bulb was used to heat the box in which dialysis was carried out. The diffusate and the dialysate were boiled down or made up to the original volume.

The active materials were tested for stability by adjusting the pH of portions of the extracts to values ranging from pH 3.0 to pH 10.0 and then heating in the autoclave at 15 lbs. pressure for one hour. Extracts were also boiled for 24 hours

in the presence of five per cent sulfuric acid in an attempt to remove tannins (Harrison and Roberts, 1939). The results gave an additional test of stability to acid.

In all cases the pH of the extracts or of the fractions was adjusted to approximate neutrality in order to avoid changing the pH of the medium or precipitation of the casein upon addition of the extract.

Results

A. Supplementary Factors

None of the strains of Tetrahymena gave growth in the vitamin-free casein medium alone or with the addition of various known growth factors. Upon the addition of 0.08 per cent of a water extract of yeast (Harris) or a water extract of crude casein good growth was obtained. This demonstrated that the casein was available to the ciliates and that the failure to grow was due to a lack of some substance, although the possibility that some toxic substance was neutralized should be kept in mind.

It was then found that when the basic medium was made up with the inorganic salt solution of Barker and Taylor growth of the ciliates occurred in the third transplant without the addition of any supplement. This growth was, however, extremely slow, taking a week or ten days to reach a maximum density of about 1000 organisms per ml., which was far below that in controls in crude casein or vitamin-free casein supplemented with hay or alfalfa extract. Addition to the basic medium of thiamine, riboflavin, nicotinic acid or pyridoxine either alone or in combination made little or no difference either in the rate or the density of growth. The same may be said of pantothenic acid, p-aminobenzoic acid, uracil, pimelic acid, i-inositol, and biotin methyl ester. These results indicate that some unknown factor (or factors) is required for the maximal growth of Tetrahymena, since crude casein gave far better growth than the basic medium supplemented by any or all of the known compounds mentioned above. The fact that transplantable growth occurs in the unsupplemented vitamin-free casein indicates either that the ciliates are capable of a slow synthesis of all their supplementary requirements or that the casein still contains traces of the required factors. The latter explanation seems more probable in view of the difficulty of obtaining chemically pure proteins. Therefore, until a medium of chemically known composition or one composed entirely of synthetic compounds can be formulated the question of the absolute requirement for various growth promoting substances will have to remain open.

It is clear, however, that for maximal growth the ciliates must be supplied with an outside source of unknown factors. These factors were found to be present in yeast, egg yolk, milk, timothy hay and alfalfa as well as meat (e.g. proteose-peptone). The animal sources were much lower in their content of growth promoting material than the plant sources. The former also contain a much larger proportion of protein material. For these reasons work was chiefly confined to the plant materials, especially since one of the aims was to obtain protein-free extracts of the growth promoting material in order to study the nitrogen requirements of the ciliates.

Preliminary experiments with a water extract of yeast had indicated that treatment with lead acetate gave an active precipitate. The procedure when tested on

extracts of hay or alfalfa gave precipitates which were much reduced in activity while the filtrates were usually inactive. A recombination of the two fractions gave growth very nearly equal to that of the controls (Table I). The indications are that some of the preparations may be slightly toxic, but, more important, that there are at least two factors present in hay or alfalfa which are necessary for the

TABLE I

Supplements	0	I	II	A or H
0	0	++	0	++++
I			+++ or ++++	

I—factor I
II—factor II
A—untreated alfalfa extract
H—untreated hay extract.

maintenance of growth at a maximal rate. For convenience the substance present in the material precipitated by lead acetate will be referred to as factor I and the material present in the filtrate as factor II.

It was also found that neither factor I nor factor II could be replaced by any one of the known growth supplements nor by a mixture of all the ten tested. This is further evidence for the existence of two substances of unknown structure necessary for maximum growth.

Further purification or a better separation of the two factors was attempted unsuccessfully by precipitation with lead acetate from an alkaline solution. Reprecipitation of the fractions with lead acetate was also unsuccessful, since the products gave evidence of greatly increased toxicity, possibly due to the increased phosphate concentration. In all these preparations protein was found to be present in the filtrate fraction and since both fractions are required for growth, the method is not useful for the removal of protein.

Ferric oxide hydrosol has been used as a protein precipitant. When tested on alfalfa and hay extracts it was found to behave similarly to lead acetate. There was a separation into two fractions, both required for optimum growth. The precipitate contained factor I and the filtrate contained factor II as well as protein (Table II).

The results with hay extract and ferric oxide hydrosol are similar to those given above for alfalfa extract but the separation is not so clear cut. A reprecipitation of the iron hydrosol fractions with lead acetate gave a more complete separation, but there was evidence of an increased toxicity of the fractions.

The material precipitated by sulfuric acid in preparation of the extracts for the addition of phosphotungstic acid was found to be inert whether alone or in the presence of either factor I or factor II. Upon the addition of phosphotungstic acid there was no clear separation into two active fractions and no removal of protein without appreciable loss of activity.

TABLE II

Supplements	0	I	II	FeI	FeII	A
0	0	++	+	±	±	++++
I			+++±		++++	
FeI			+++±		++++	

FeI—iron hydrosol procipitate
FeII—iron hydrosol filtrate
Other symbols as in Table I.

At this point the possibility that carbohydrate might be concerned in the activity of these fractions arose. Since all the reducing sugar in the extracts was found in the filtrate fraction from the lead acetate treatment, this fraction was treated with copper sulfate and calcium hydroxide. A complete removal of the reducing sugars but not of the protein in the preparations was possible without appreciable loss in activity. This treatment may be valuable in the further purification of the factors, since other inert materials appeared to be removed with the sugars.

Since heavy metals failed to remove protein, extractions with various organic solvents was tested as a means of obtaining protein-free preparations. Extracts prepared with ether, acetone, alcohol and acetic acid were found to be inactive or even toxic.

Dialysis also failed to remove protein or protein breakdown products. Some nitrogenous material of this nature was found to be freely diffusible as were both factor I and factor II. The dialysate in all cases was inert; all activity was found in the diffusate. The fact that there was some loss of activity from the extracts during dialysis led to the conclusion that one of the factors is destroyed by light. When the electric light bulb used to heat the box in which dialysis was carried out was screened the loss of activity did not occur. This may be correlated with the progressive loss of activity of extracts exposed to ultraviolet radiation for increasingly longer intervals (Kidder and Dewey, 1942, mistakenly state that factor I is affected by the irradiation). The results indicate that factor II is destroyed by light. This is evidence also for the organic nature of the growth promoting material.

Adsorption upon activated charcoal or Fuller's earth followed by selective elution is a well known means of purification of growth factors. When this method was tested it was found that both factors, as well as protein, are readily adsorbed upon Norit and much less readily upon Super Filtrol. The filtrate after the Norit treatment was completely inert. Both factors (as well as the protein) appear to be eluted by alkaline alcohol (Table III). The elutions from Super Filtrol were more successful, possibly because the materials are less strongly adsorbed. Although this method may be useful in the purification of the separate fractions after precipitation with lead acetate, it was discarded as a means of protein removal.

The tests of the stability of the growth substances to heat at various pH values showed that there was no loss of activity in alfalfa extracts in either acid or alkaline

TABLE III

Supplements	0	I	II	E	A
0	0	++	0	+++	++++
I			+++	++++	
II				++++	
F	+++	++++	+++±	++++	

F—filtrate after adsorption
E—Eluate from Super Filtrol.

solution. On the other hand proteose-peptone treated at an alkaline pH and used to supplement casein was almost inert. By testing it was found that this was due to a loss of factor I during the treatment (Table IV). Factor I from animal sources therefore appears to be heat-labile. The loss of activity in heat-treated proteose-peptone is not due to the destruction of thiamine. The more drastic treatment such as that described for the removal of tannins destroyed activity entirely, which is further evidence for the organic nature of the supplements.

TABLE IV

Supplements	0	I	HA	HPP	A
0	0	++	++++	±	++++
I				++++	
II	±	++++		+	
Thiamine	±	++		+	

HA—alfalfa extract heated at high pH
HPP—proteose-peptone heated at high pH.

The last method tested for the removal of protein was precipitation with organic solvents. Both the whole extracts and the fraction (filtrate) containing the protein after lead acetate precipitation were treated by the addition of alcohol up to a concentration of 75 per cent. This method was successful in the removal of protein from the hay extracts but not from the alfalfa extracts. The precipitates obtained from hay were inert and the activity of the filtrates was unaffected (Table V). Whole hay extract treated in this manner was used in the experiments on nitrogen nutrition to be described later.

The effect of the addition of barium hydroxide plus alcohol was tested on the alfalfa extract in the hope of precipitating the protein. It was found, however, that 75 per cent alcohol alone precipitated some of both factors along with some of the protein. The addition of barium then had an effect similar to that of lead acetate in that there was a partial separation of the two factors.

Acetone was next considered as a means of removing protein from alfalfa extracts. Its behavior was similar to that of alcohol in that the active substances were precipitated along with the proteinaceous material, the amount increasing as the concentration of the acetone was increased. At 80 per cent factor I was largely precipitated and factor II to a smaller extent. Since protein-free extracts could be obtained readily from hay, the work on alfalfa was discontinued even though it is a richer source of growth-promoting material.

TABLE V

Supplements	0	I	II	IIp	IIf	H	Hp	Hf
0	0	++	±	±	±	++++	±	++++
I			++++	++±	++++			

IIp—precipitate from alcohol treatment of factor II fraction
IIf—filtrate from the same
Hp—precipitate from alcohol treatment of hay extract
Hf—filtrate from same.

From the above the properties of the two factors may be summarized as follows: soluble in water, moderate concentrations of alcohol and in low concentrations of acetone; insoluble in ether; stable to heat (plant sources only in the case of factor I); dialyzable through cellophane; readily adsorbed on charcoal and less readily upon Super Filtrol; eluted by ammoniated alcohol. Factor II differs from factor I in that the former is not precipitated by the salts of heavy metals and appears to be destroyed by irradiation.

When either Harris gelatin or Eastman de-ashed gelatin was used as a basic medium (1.5 per cent solution) the results obtained were similar to those obtained with casein as a basic medium, except that the population density was smaller. In the Harris gelatin alone slight but transplantable growth, which was somewhat improved upon the addition of inorganic salts, was obtained. The addition of thiamine, riboflavin, pantothenic acid or biotin gave little or no improvement in growth, while the addition of hay extract gave a considerable increase in the rate and density of growth.

With de-ashed gelatin the addition of inorganic salts was necessary and in their presence without the addition of supplements slight transplantable growth occurred. The addition of thiamine or ribroflavin (0.0001 mg. per ml.) or both together gave no improvement in growth. Growth was increased only upon the addition of both hay extract and riboflavin to the medium.

B. Nitrogenous Nutrition

The experiments to be described below are exploratory in nature and have served chiefly to suggest further experiments and modes of attack upon the problem. Some of the work of earlier investigators was repeated in the hope that the use of an adequately supplemented medium might give better results than had been obtained.

The first experiments were carried out upon completely hydrolyzed casein. Such a medium was chosen in the hope of shedding more light upon Lwoff's (1932) hypothesis that polypeptides are required for growth. Acid digestion was used because complete hydrolysis by enzymatic means is difficult if not impossible and alkaline hydrolysis has a destructive effect on many of the amino acids. No growth occurred in the acid hydrolysate even in the presence of what was considered to be adequate supplementation. Attention was then turned to a solution of pure amino acids, also supplemented with protein-free hay extract. This solution was prepared using the ten amino acids found by Rose (1938) to be necessary for the nutrition of the mammal. The amino acids were present in the concentrations found in a one per cent solution of casein. Again no growth occurred in this solution or in various dilutions of it.

Such solutions have an osmotic pressure lower than that of salt solutions readily tolerated by the organism. The explanation for the lack of growth must, therefore, be sought elsewhere. Three other possible explanations for the lack of growth are, *a*) that one (or more) amino acid required for the growth of the organism is lacking, *b*) one or more of the amino acids present is toxic or inhibitory, and *c*) that the organism requires nitrogen in the form of polypeptides.

The possibility of the toxicity of the amino acids was considered first. These experiments were to be correlated with others using gelatin as a basic medium and supplemented with one or more of the amino acids known to be lacking from this protein. For this reason those particular amino acids were added to casein as well as to gelatin in the concentrations in which they are found in a one per cent solution of casein. The results in the two media were strikingly different. With casein it was found that the addition of free amino acids had little or no effect on growth. In the case of gelatin (one per cent vitamin-free gelatin Harris) definite inhibition of growth was found in those cultures containing valine, tyrosine or isoleucine. Hydroxyglutamic acid was not then available. When tryptophane was added to the gelatin there was a large increase in the growth and media containing tryptophane in addition to valine or tyrosine gave better growth than similar media lacking tryptophane. It was found that decreasing the concentration of these amino acids to 0.0025 per cent improved the growth in all cases, although tyrosine, valine and isoleucine still showed inhibition of growth. In all cases the media contained protein-free hay extract.

These experiments were repeated in tube cultures three or four times, but in order to check the observations cultures were incubated in Kidder culture flasks and the growth followed by making counts at intervals of 12 hours. With casein plus 0.01 per cent tyrosine it was found that the population density at the end of the phase of logarithmic growth (48 hours) was 81,000 organisms per ml. and without tyrosine 75,500 per ml. The figures in the case of tryptophane were quite similar, 84,000 and 70,000 respectively, with and without 0.01 per cent tryptophane. The generation times did not differ significantly in any of the media. The differences in population density in these media represent less than one division per ciliate and are not regarded as being of statistical significance.

With one per cent gelatin as a basic medium it was found that the addition of 0.01 per cent tyrosine, valine or isoleucine gave maximum populations of only a few hundred organisms per ml. Gelatin alone gave 15,000 per ml. and with the addition of tryptophane a maximum of 90,000. When the concentration of added

amino acid was reduced to 0.0025 per cent tyrosine gave a maximum of 12,000; valine, a maximum of 7,100; isoleucine, a maximum of 900 and tryptophane a maximum of 91,000 organisms per ml. In all cases the generation time was lengthened.

When two per cent gelatin was used a population of 80,000 organisms per ml. was obtained and when 0.002 per cent tryptophane was added the maximum was 230,000 organisms per ml. In this case the amino acid caused no decrease in interdivisional time.

The above results indicate that certain amino acids are detrimental to the growth of Tetrahymena, but suggest that this inhibition is reduced or absent in the presence of large protein molecules such as casein, or in the presence of tryptophane. The growth in two per cent gelatin with and without tryptophane leads plausibility to the theory that large protein molecules or a sufficient concentration of smaller protein molecules in some way decreases the inhibitory effect of free amino acids upon the ciliates. Time did not permit the testing of the more toxic amino acids with the higher concentration of gelatin.

Silk peptone, the only other incomplete protein preparation readily available, gave such good growth when supplemented with hay extract that it was not used as a basic medium for the study of amino acid requirements.

Discussion

Of the four types of substances generally accepted as being required for growth of an organism (inorganic salts, supplementary substances, carbon and nitrogen compounds) it is evident that *Tetrahymena geleii* requires inorganic salts (Hall, 1942; Hall and Cosgrove, 1944 and data presented here), supplementary factors and an organic source of nitrogen which supplies the needs for both carbon and nitrogen. The requirement for a source of carbon separate from the source of nitrogen has never been demonstrated.

At present the question of the supplementary factor requirements of Tetrahymena remains unsettled. So far the claims that thiamine is a growth factor (i.e. an absolute requirement for growth) have not been substantiated. Indeed under certain conditions it is not even to be regarded as a growth stimulant (Kidder and Dewey, 1942). The work of Hall and Cosgrove (1944) fails to refute this claim.

Hall (1942) claims that riboflavin is also a growth factor for Tetrahymena (*Colpidium campylum*). This work could not be confirmed, although stimulation of growth could be obtained with both thiamine and riboflavin under certain conditions. In any case the growth stimulation obtained with the two unknown factors described above is far more powerful than that caused by either of these compounds.

Elliott (1935b) reports an increase in the maximum population density of cultures when pantothenic acid was added to tryptone media. Since he was using a crude preparation of pantothenic acid this effect may have been due to other substances in the preparations. Pantothenic acid has subsequently been found to have have no effect on growth when added to a casein medium. So far as can be determined from the data published (Hall, 1939; 1942) pimelic acid has no "acceleratory" effect on growth. The effect appears to be due to the introduction of inorganic salts.

Certain secondary effects have been attributed to thiamine, riboflavin and other known growth substances (Hall, 1940a; Hall and Shottenfeld, 1941; Baker and

Johnson, 1941). These are concerned with the death and decline phases of growth and are not of immediate interest here. It is of more importance to the problem under consideration that none of the known growth-promoting substances will permit maintenance of growth at the maximum rate and of a maximum density. For such growth at least two substances of unknown nature are required. Whether or not some of the known compounds may also be required for such growth cannot be decided until pure preparations of these substances and a basic medium known to contain no growth supplements are available. The use of purified gelatin for a basic medium may give information of some value, but it is not truly suitable, since its use introduces the complication that it does not satisfy a possible requirement for one or more of the amino acids it lacks. Although the so-called vitamin-free casein is not altogether ideal because it appears to contain traces of growth promoting materials, it is nevertheless an adequate basic medium for a study of growth stimulation. Unsupplemented, the growth it supports is far from maximal.

The fact that growth of these ciliates can be obtained in gelatin solutions when properly supplemented, as pointed out by Hall (1942), would indicate that the ciliate requires for growth none of the amino acids lacking from that protein. In other words *T. geleii* must synthesize tryptophane, valine, hydroxyglutamic acid, isoleucine and possibly tyrosine unless its protoplasm does not contain these amino acids. This latter hypothesis seems most unlikely especially in view of the fact that tryptophane increases the growth so remarkably. It is difficult to explain however, why tryptophane increases the maximum concentration of organisms obtained rather than the growth rate.

The failure of other investigators to obtain growth with solutions of amino acids or incomplete proteins supplemented with amino acids is now understandable. In some cases (Lwoff, 1932; Elliott, 1935b) the media contained none of the supplementary factors now known to have a profound effect on growth. Nor can the claims of Hall and Elliott (1935) regarding the effects of certain amino acids be regarded as conclusive, since their results were expressed as x/x_0. As Kidder (1941b) has pointed out, this method of representation may give an entirely false conception of the results obtained. Another source of possible error in the earlier work may lie in the use of concentrations of amino acids which may now be regarded as inhibitory to growth. It is possible that this difficulty may be overcome by the adsorption of amino acids upon inert colloids and by the use of tryptophane, which appears to decrease the toxicity of other free amino acids.

In view of the inhibitory effect of free amino acids and of the ability of *T. geleii* to grow in an incomplete protein such as gelatin, it is difficult to understand the report of Kline (1943) that *T. geleii* (*Colpidium striatum*) will grow in a solution of 15 amino acids with the addition of various supplements. It is possible that the explanation lies in the fact that different strains of *T. geleii* were used.

No definite decision can as yet be made between the three suggested possibilities for the lack of growth of *T. geleii* in amino acid solutions. The evidence on hand, however, suggests that the factor of toxicity of free amino acids is of some importance. This effect, rather than a requirement for polypeptides, is a possible explanation for the decreasing growth obtained by Lwoff (1932) as the degree of hydrolysis of the medium used was increased. This would be true whether or not adequate supplements were present.

SUMMARY

1. The known growth promoting substances alone or in various combinations are not sufficient for the growth of *Tetrahymena geleii* at a maximal rate and density.

2. At least two unknown substances (factor I and factor II), present in both plant and animal materials, are required for such growth.

3. Factor I is distinguished from factor II by the fact that the former is precipitated by heavy metal salts while the latter is not.

4. Active protein-free preparations of these factors may be prepared from extracts of timothy hay by treatment with ethyl alcohol.

5. Growth of the ciliate could not be obtained in acid digests of casein or in solutions of free amino acids supplemented with the protein-free extract.

6. Tyrosine, valine, and isoleucine were found to be inhibitory in the presence of gelatin, but not in the presence of casein.

7. A large increase in the population density occurred in the presence of tryptophane and gelatin but not with tryptophane and casein.

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THIAMINE AND TETRAHYMENA

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Since the publication of our paper on the biosynthesis of thiamine by *Tetrahymena geleii* and *T. vorax* (Kidder and Dewey, 1942) we have expanded our investigations with regard to the basic medium employed. In the earlier work "vitamin-free casein" (Harris, highest chemical purity) was used exclusively as a base. This protein in a one per cent solution with added salts (modified Osterhout's solution) gave "very little growth" either alone or when any or all of the following vitamins were added: thiamine, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, pimelic acid, i-inositol, uracil and p-aminobenzoic acid. Optimum growth resulted when extracts of plant leaves were added (Dewey, 1941; 1944). Even after the extracts were heated for one or more hours at 123° C. at pH 10–11.5 (to destroy thiamine) it was found that optimum growth again resulted without the addition of thiamine. It was further shown that thiamine had been synthesized by *T. geleii* by testing with *Glaucoma scintillans,* an organism dependent upon exogenous thiamine. A tentative conclusion was reached that there was, in the plant extracts, a factor which in some way made it possible for Tetrahymena to synthesize thiamine. This tentative factor was designated factor S.

It has recently been stated by Hall and Cosgrove (1944) that there was no evidence for the biosynthesis of thiamine by Tetrahymena on the basis that "vitamin-free" casein contains appreciable amounts of thiamine. They were able to obtain transplantable growth, using their strain of *T. geleii* (*Glaucoma piriformis*), in unsupplemented one per cent salted casein but not in heat- and alkali-treated salted casein unless thiamine was added. They believe that our low growth was due to a lack of minerals in the media used. Their criticism regarding the mineral factors is justified, as it was not clearly stated in our paper that salts (modified Osterhout's solution) were added. Inorganic salts were mentioned only in "substances used in the preparation of media."

The following experiments, modified to insure thiamine-free media, confirm our earlier results and again show that *Tetrahymena geleii* (strain W) is entirely independent of an exogenous source of thiamine for indefinitely transplantable growth. Quantitative results are being presented for the first time in this connection.

Material and Methods

Pure culture (bacteria-free) *Tetrahymena geleii* (strain W) was used exclusively in the present study. This is the strain which was used by us on previous occasions (Kidder, 1941a; Dewey, 1941; 1944; Kidder and Dewey, 1942). All experiments were carried out in chemically clean Pyrex tubes or flasks and aseptic technique was employed throughout. All media were prepared with water distilled twice over permanganate in an all-Pyrex still. The following substances were used:

casein (Harris, highest chemical purity); gelatin (Harris, selected grade, refined, vitamin-free); alfalfa leaf meal (Denver Alfalfa Milling and Products Co.); thiamine hydrochloride (Hoffman-LaRoche); riboflavin and l-tryptophane (Merck and Co.); inorganic salts (Baker and Adamson). All media were sterilized by autoclaving.

Base media

1. Heated casein—A two per cent solution (treated as described by Dewey, 1944) of casein was autoclaved for one hour at pH 10. After cooling and neutralizing the concentration was adjusted to one per cent or to 0.5 per cent. This medium is always quite turbid and a precipitate settles out upon standing, so the available casein is considerably reduced from the figures given.

2. Filtered heated casein—Heated casein was allowed to cool, the pH adjusted to 6.8, and the precipitate removed by filtration through a Buchner filter with the aid of Celite (Johns-Manville). The filtrate was then diluted to what would be 0.5 per cent (calculated on the original casein). This gave a light straw colored clear solution which again precipitated slightly upon final sterilization.

3. Casein hydrolysate—Two per cent casein was refluxed 22 hours in a 24 per cent solution of H_2SO_4. The sulphate was removed with $Ba(OH)_2$. The resulting hydrolysate was biuret negative. This hydrolysate was used in a 0.5 per cent concentration (calculated from the original amount of casein used).

4. Gelatin—This was used in a two per cent solution.

5. Heated gelatin—Four per cent gelatin was autoclaved one hour at pH 10. After cooling the pH was adjusted to 6.8 and the solution was diluted to a concentration of two per cent.

6. Gelatin hydrolysate—Four per cent gelatin was refluxed for five hours in a 24 per cent solution of H_2SO_4. The sulphate was removed by $Ba(OH)_2$. This hydrolysate was biuret negative and was used in one per cent concentration (calculated from the original amount of gelatin used).

Alfalfa extract was prepared as described previously (Kidder and Dewey, 1942). After heat and alkali treatment at pH 10 it was adjusted to pH 6.8 and added in a dilution of 1 : 10 final concentration. This dethiaminized extract is designated A.

Thiamine hydrochloride was added where indicated in the concentration of one microgram per ml. of medium.

To all of the media used in the following experiments were added just before sterilization the following inorganic salts (Hall and Cosgrove, 1944): 0.02 per cent $MgSO_4 \cdot 7H_2O$; 0.02 per cent K_2HPO_4; 0.01 per cent $CaCl_2 \cdot 2H_2O$; 0.00025 per cent $FeCl_2 \cdot 6H_2O$; 0.00001 per cent $MnCl_2 \cdot 4H_2O$; 0.00001 per cent $ZnCl_2$. To all media was also added 0.1 microgram per ml. of riboflavin. Tryptophane was added to the hydrolysed casein (to compensate for loss in hydrolysis) and to all gelatin and gelatin hydrolysates to a concentration of 0.0025 per cent. Experiments with amino acid mixtures now being conducted show that tryptophane is essential to the growth of Tetrahymena. All media were used at pH 6.8–6.9.

A number of preliminary experiments were carried out with each medium in tubes in serial transplants. Each tube contained five ml. of medium. All tube series were inoculated with a bacteriological loop delivering approximately 0.008 ml. Tube series were grown through at least three transplants before any conclusions

were drawn, this to eliminate the possibility of carry over of medium from the stock cultures. Tube cultures were incubated at room temperature and transplants were made every 48 hours, except where very slow growth occurred in the early transplants, where longer times were allowed.

The quantitative studies were made using the culture flasks described earlier (Kidder, 1941b). These flasks contained 100 ml. of media. Inoculations were made from third transplant tubes of like media so that the flask cultures represent fourth transplant series. Sterile serological pipettes were used for the inoculations and from 0.1 ml. to 0.5 ml. was added, depending upon the density of the population in the tube from which the inoculation was made. After the first few experiments inoculations were made from cultures within the exponential growth phase and the inoculations were calculated to give an initial count of as near 100 cells per ml. as possible. Flask cultures were incubated at 24.5° C. All flask experiments were repeated at least once.

Our method of counting cells from culture has been described elsewhere (Kidder, 1941b), but it should be noted here that this method gives only viable counts, hence our population counts tend to be lower in the stationary phase and phase of decline than where methods involving the counting of killed cells is employed. These differences are well illustrated in the work of Johnson and Baker (1943).

Generation time (g) was calculated by the use of the formula

$$g = \frac{t \log 2}{\log b - \log a}$$

where t = the time in hours during which the population has been increasing exponentially, a = the number of cells per unit volume at the beginning, and b = the number of cells at the end of time, t.

Experimental

Population Studies

Casein and casein hydrolysate—When a solution of casein is adjusted to pH 10 and autoclaved for one hour to render it thiamine-free, a number of changes take place which make it very inferior to unheated casein as a basic medium for Tetrahymena. Hall and Cosgrove (1944) state that factors in addition to thiamine must have been destroyed, because even upon the addition of thiamine poor growth resulted. With our strain of *T. geleii* heat treated casein plus thiamine (also salts and riboflavin, as mentioned above) inhibited growth even in the first transplant, and second transplants were almost invariably negative. In no case was growth obtained in the third transplant. However, if the insoluble precipitate resulting from such treatment is filtered off and the concentration (originally one per cent before filtration) is reduced by one-half then low but transplantable growth results. The addition of thiamine has no significant effect upon the generation time, length of the logarithmic phase, maximum yield or survival up to the limit of our experiment (Table I; Fig. 1). This indicates that the heat treatment has produced toxic substances which, when reduced in concentration do not inhibit growth entirely. It also shows that Tetrahymena can reproduce without an exogenous source of thiamine.

TABLE I

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days
Heated casein 0.5 per cent + A	5.02	18,000	160,000	61,000
Heated casein 0.5 per cent + A + B_1	4.80	17,500	182,000	82,000
Filtered heated casein	8.35	1,600	5,500	2,000
Filtered heated casein + B_1	8.96	1,800	8,000	3,200
Filtered heated casein +A	4.43	42,000	100,000	57,000
Filtered heated casein + A + B_1	4.27	38,000	110,000	86,000

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts and riboflavin (0.1 micrograms/ml.).

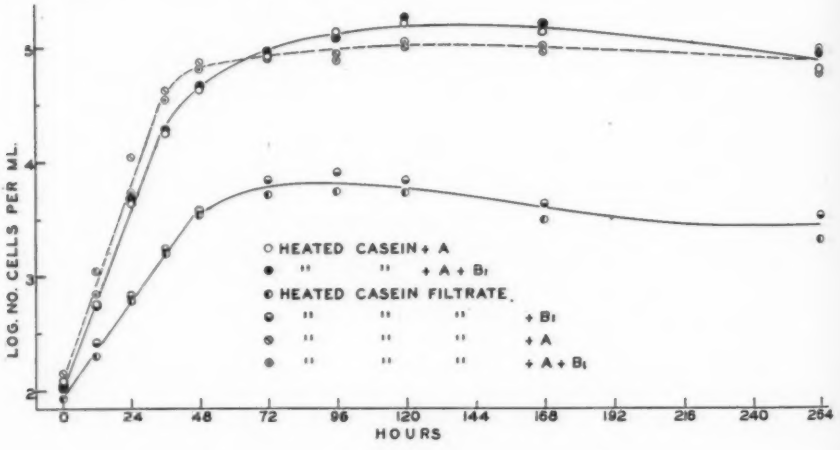

FIGURE 1

When dethiaminized alfalfa extract is added to the heated casein or to the filtered heated casein the response is striking. Rapid growth now occurs in the heated casein while in the filtered heated casein the generation time is reduced by nearly one-half and the population at the end of the logarithmic growth phase is increased from around 1,600 to over 40,000 per ml. The maximum yield is increased from about 5,000 to approximately 100,000 per ml. and a much higher population is maintained for at least 11 days (over 50,000 as compared to 2,000 per ml.) (Table

I; Fig. 1). This would seem to indicate that, in addition to supplying stimulatory factors (Dewey, 1944) and the synthesizing factor, the alfalfa extract counteracts the toxic effects of the heat treatment on the casein. There is indication in the shape of the growth curve that the greater toxicity of the heated casein has not been as successfully counteracted as that of the filtered heated casein. The generation time is approximately 0.5 hour longer in the former and the population begins to fall off sooner. The maximum yield, however, is higher (160,000 as compared to 100,000 per ml.) in the unfiltered casein. This last may be due to the higher concentration of available protein.

TABLE II

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days
Casein hydrolysate 0.5 per cent + A	4.27	50,000	122,000	49,000
Casein hydrolysate 0.5 per cent + A + B_1	4.36	31,000	191,000	94,000
Heated casein hydrol. 0.5 per cent + A	4.37	29,000	182,000	54,000
Heated casein hydrol. 0.5 per cent + A + B_1	4.46	32,000	171,000	73,000

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

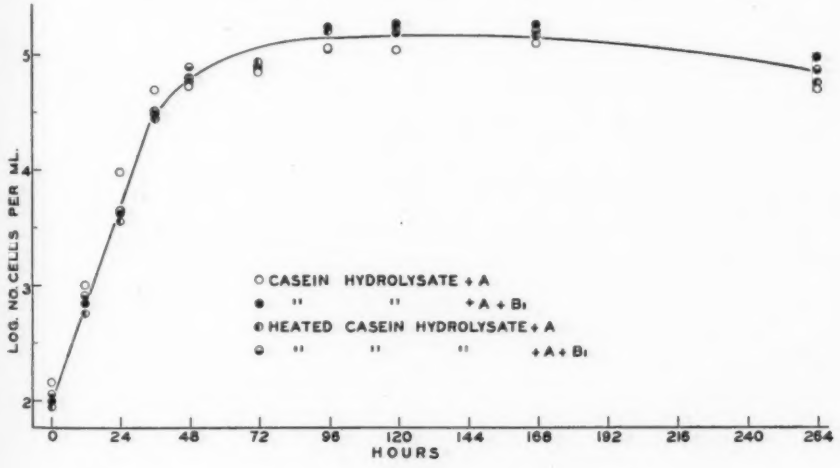

FIGURE 2

The addition of thiamine to either the heated casein or filtered heated casein plus dethiaminized alfalfa extract has very little effect. The cultures maintain a slightly higher level at 11 days duration but the shape of the growth curves are almost identical.

When a 0.5 per cent solution of a biuret negative casein hydrolysate plus 0.0025 per cent l-tryptophane was used as a basic medium it was found that growth was impossible beyond the first transplant, even when thiamine was added. Inasmuch as unheated casein and filtered heated casein give slow but indefinitely transplantable growth the acid hydrolysis must have destroyed some factor or factors, other than thiamine, necessary for growth. Excellent growth resulted, however, when de-

TABLE III

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days	Size of cells at 11 days (av. 20 measurements)
Gelatin 2 per cent	5.68	2,600	12,000	2,100	$22\mu \times 16.5\mu$
Gelatin 2 per cent + B_1	5.59	2,400	67,000	31,000	$91.5\mu \times 34\mu$
Gelatin 2 per cent + A	3.21	18,000	140,500	47,000	$51\mu \times 24\mu$
Gelatin 2 per cent + A + B_1	3.22	17,200	161,000	52,000	$86.5\mu \times 22\mu$

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

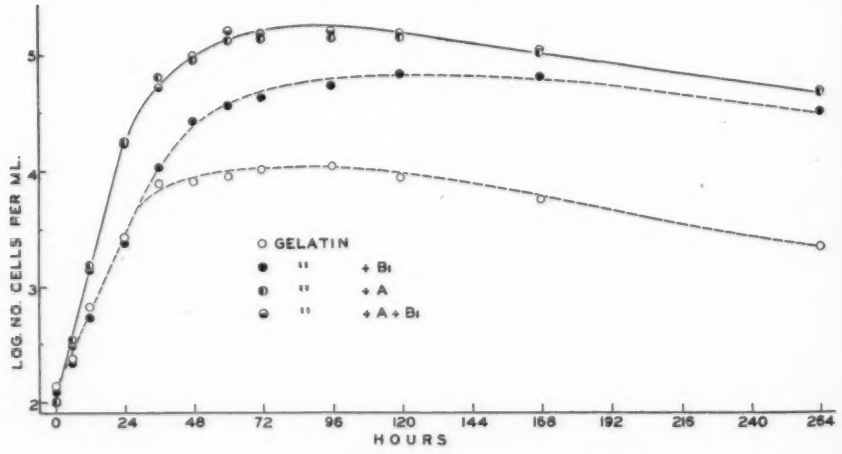

FIGURE 3

TABLE IV

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days	Size of cells at 11 days (av. 20 measurements)
Heated gelatin 2 per cent	5.31	2,750	11,700	200	$20\mu \times 16\mu$
Heated gelatin 2 per cent + B_1	5.72	2,000	68,500	44,000	$85\mu \times 30\mu$
Heated gelatin 2 per cent + A	3.08	19,500	82,000	40,000	$47\mu \times 20\mu$
Heated gelatin 2 per cent + A + B_1	3.42	17,500	96,000	72,000	$89\mu \times 36.5\mu$

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

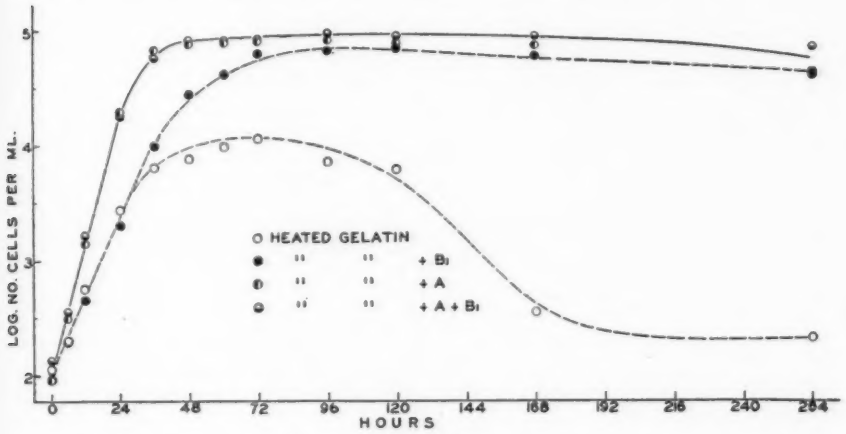

FIGURE 4

thiaminized alfalfa extract was added, and again the addition of thiamine had no effect except upon survival (Table II; Fig. 2).

Gelatin and gelatin hydrolysate—Gelatin Harris is a purified product, according to its manufacturers. It was found, however, that fair growth could be maintained in a one per cent or a two per cent solution provided tryptophane was added. The generation time was about 5.5 hours, the population at the end of logarithmic growth about 2,500, the maximum yield about 12,000 and at the end of 11 days the population was approximately 2,000 per ml. The addition of thiamine had no effect on the generation time or the length of the logarithmic phase but the maximum yield was increased to 67,000 per ml. while at the end of 11 days over 30,000 ciliates per ml. were present (Table III; Fig. 3). This might indicate that enough thiamine

was present for limited growth in the unsupplemented gelatin and that added thiamine was necessary for the increased maximum yield and higher survival.

When dethiaminized alfalfa extract is added to gelatin plus tryptophane the generation time is reduced to a little over three hours, the population at the end of logarithmic growth is increased to about 18,000, the maximum yield is increased to over 140,000 and the population at the end of 11 days is increased to 47,000 per ml. (Table III; Fig. 3). This shows the stimulatory effect of the alfalfa factors. The

TABLE V

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days	Size of cells at 11 days (av. 20 measurements)
Gelatin hydrolysate 1 per cent + A	4.22	57,500	195,000	8,500	50.5μ × 27μ
Gelatin hydrolysate 1 per cent + A + B_1	4.26	51,500	208,000	10,000	99μ × 41μ
Heated gelatin hydrolysate 1 per cent + A	4.59	54,500	160,000	11,000	42.5μ × 19μ
Heated gelatin hydrolysate 1 per cent + A + B_1	4.62	31,000	321,000	9,200	78μ × 35μ

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

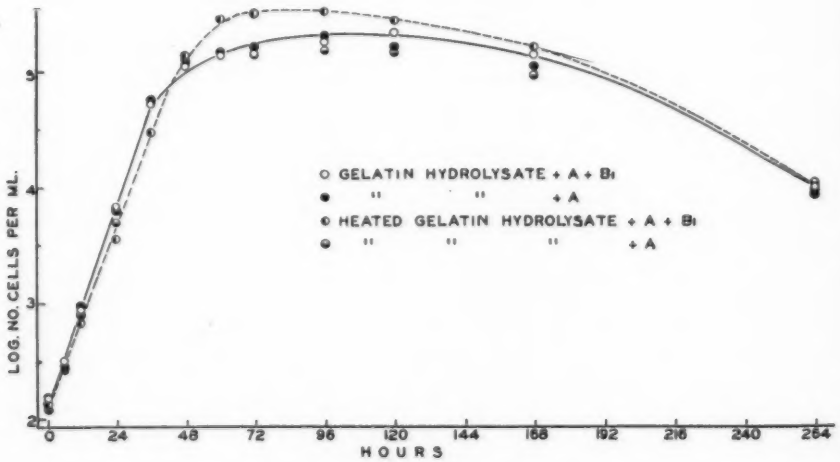

FIGURE 5

addition of thiamine to gelatin-tryptophane plus dethiaminized alfalfa extract had no significant effect.

When dethiaminized gelatin plus tryptophane is used the only apparent difference from unheated gelatin is in the survival. The ciliate population in heated gelatin begins to decrease more rapidly until at the end of 11 days only about 200 per ml. remain (Table IV; Fig. 4). In eighth transplant tube cultures viable ciliates were found after 30 days. There can be no question that *Tetrahymena geleii* (strain W) does not require an exogenous source of thiamine for limited growth in this medium. Tube cultures were carried through six transplants using glass wool instead of the usual washed and bleached cotton for stoppers, in order to be sure that no trace of thiamine could enter the medium from the few cotton fibers which sometimes drop from the stoppers. All factors required for thiamine synthesis by the ciliates must be present in limited amounts in gelatin and must withstand the rigorous heat and alkali treatment. The addition of thiamine or alfalfa extract or both to heated gelatin produced about the same results as when added to unheated gelatin (Table IV; Fig. 4).

Acid hydrolysis of gelatin destroys factors necessary for the growth of *Tetrahymena geleii* (strain W) for in no case was growth maintained beyond the initial transplants without the alfalfa factors. This is comparable with the casein hydrolysate. The addition of thiamine had no effect.

Excellent growth resulted when dethiaminized alfalfa extract was added to gelatin hydrolysate plus tryptophane. Best growth was obtained in a one per cent solution of the hydrolysate although two per cent gave only slightly lower growth. The addition of thiamine to the hydrolysate plus alfalfa extract had no effect (Table V; Fig. 5).

Heating the gelatin hydrolysate at 123° C. for one hour at pH 10 caused slight changes of doubtful significance. Again excellent growth occurred upon the addition of dethiaminized alfalfa extract and again the addition of thiamine had no effect on population growth (Table V; Fig. 5). This again demonstrates that these ciliates do not require exogenous thiamine for continued growth. In tube cultures the tenth transplant (in heated gelatin hydrolysate plus tryptophane and dethiaminized alfalfa extract) behaves like the first transplant, and with the technique employed there can remain no effective carry over from the stock solution.

Ciliate Size Relations

The above discussion has been concerned only with population growth. The individual cells vary in size and shape to a great extent depending upon the type of medium employed. This variation is never apparent, however, during active multiplication, but only during the stationary phase and the period of population decline. Detailed observations and measurements were made on cultures based on gelatin and gelatin hydrolysate. The differences noted were due to the presence or absence of the alfalfa extract or of thiamine and not to the nitrogen source.

When the ciliates have grown for from 8 to 11 days in a medium lacking both the alfalfa factors and thiamine (outside supply) they become very small (Tables III and IV) and evenly piriform. The protoplasm appears somewhat dense and the motion of the ciliates is very reduced. The small size can be detected with the naked eye in tube cultures. These small ciliates are perfectly viable, however, and normal cultures result upon transplantation into fresh medium.

When thiamine is added to the medium the ciliates increase in size during the stationary period until at the end of 11 days (the time when measurements were taken) they may be as much as 100 μ in length (Tables III and IV). Most of them are flattened and irregular in shape, are fairly active and quite transparent.

When the alfalfa extract is added to the base medium the ciliates in the stationary period and period of decline are about midway in size between those in media with neither alfalfa nor thiamine and those in the media with added thiamine (Table III, IV and V). These ciliates are also flattened and transparent and are actively motile.

When both the alfalfa factors and thiamine are added together large ciliates result. These are about the size and appearance of those in cultures where only thiamine is added (Tables III, IV and V). They are more actively motile, however.

The above observations are preliminary and limited in nature but they indicate differences due to accessory factors and warrant more detailed study.

Discussion

From the results of our experiments with media based on casein and gelatin we can offer the following statements regarding thiamine and *Tetrahymena geleii* (strain W). In a dethiaminized medium of gelatin plus tryptophane or in dethiaminized casein (filtered) plus inorganic salts and riboflavin this ciliate can be serially transplanted apparently indefinitely. It appears to be able to synthesize thiamine enough for moderate to low population growth. This synthetic activity seems to be in direct ratio to the amount of some substance (factor S of Kidder and Dewey, 1942) present in small amounts in the heat-treated casein and gelatin. When thiamine is added to the gelatin medium no effect is shown during the logarithmic phase. But as the factors catalyzing the synthesis of thiamine are being depleted (end of logarithmic phase), reproduction falls off sharply in the gelatin medium alone but continues further for some two and a half divisions when outside thiamine is provided. The presence of toxic products of the heat and alkali treatment on casein makes this medium so unsuitable for the ciliates that growth is limited even with added thiamine.

The raising of the reproductive rate by the addition of "factors I and II" (Dewey, 1944) contained in the alfalfa extract counteracts the toxicity of the heat and alkali treated casein. Also large amounts of "factor S" are made available for the synthesis of thiamine over a longer period of time. Indeed this synthesis is enough to meet the requirements for rapid growth and the addition of an outside supply of thiamine has no effect, until the period of population decline.

It seems evident that the denial by Hall and Cosgrove (1944) of our previous conclusions (that *T. geleii* can synthesize thiamine) can now be dismissed. There can be no question of the thiamine-free nature of our medium. A point of some interest, however, is the fact that they obtained transplantable growth with their strain of Tetrahymena (*Glaucoma piriformis*) in "1 per cent dethiaminized casein" plus salts and thiamine but not when thiamine was omitted. Our strain (W) failed to grow in this strength either with or without thiamine. Strain differences may account for this apparent discrepancy, as we found that *T. geleii* (Hetherington strain), the one used in previous studies in this laboratory (Kidder, 1941b; Kidder and Stuart, 1939; Kidder, Lilly and Claff, 1940), is more resistant to the

toxic substance produced by the heat and alkali treatment on casein and grew in serial transplants very slowly and in low concentration in 1 per cent heated casein plus thiamine, riboflavin and salts. Another factor which must be taken into consideration is the fact that Hall and Cosgrove discarded the precipitate after decanting the supernatant fluid from heat and alkali treated 1 per cent casein. This would make their medium similar to our filtered heated casein. In view of the activity of the ciliates in heated gelatin where toxicity is less pronounced we believe the principal effect of thiamine in this case as well as in the case recorded by Hall and Cosgrove was to detoxify the medium. We have similar data with various amino acids in other types of experiments now being carried on. Had Hall and Cosgrove repeated our experiments (1942) by using dethiaminized alfalfa extracts they would not, in all probability, have stated that "the results obtained with Glaucoma piriformis afford no basis for concluding that this ciliate synthesizes thiamin. . . ."

We are in agreement with Hall and Cosgrove (1944) regarding the mineral requirements of *Tetrahymena* and accordingly the inorganic salts were always added. We do not know the specific effect of riboflavin on strain W but knowing that this vitamin is rapidly destroyed in alkaline solution by light it was thought best to add sufficient amounts to insure against its being a limiting factor.

In our previous report (Kidder and Dewey, 1942) we stated that "factor S" had not been detected in material of animal origin. It was pointed out, however, that the production of toxic substances by the heat and alkali treatment might mask the presence of "factor S." We are now of the opinion that small amounts of "factor S" are present in Gelatin Harris and Casein Harris and possibly associated with other animal proteins.

In this study we have assumed the synthesis of thiamine by Tetrahymena on the basis of transplantable growth in completely dethiaminized media. This assumption might be questioned on the basis that there is a possibility that Tetrahymena does not use thiamine in its metabolic activities. As far as we know it would then be unique among plants and animals. It must also be remembered that evidence was presented previously (Kidder and Dewey, 1942) that thiamine was actually synthesized and could be detected by the use of *Glaucoma scintillans,* a thiamine-requiring ciliate. Also in view of the real effects adequate amounts of thiamine have on the population levels and on survival (see Johnson and Baker, 1943) and on size, the inclusion of thiamine in the metabolism of this ciliate is almost certain.

One point of importance which should be discussed here is our success in obtaining growth in completely hydrolysed proteins. Lwoff (1932) found that *"Glaucoma piriformis"* would not grow in abiuretic media. He concluded tentatively that these ciliates require peptides or more complex molecules in addition to growth factors. He did not use anything which would correspond to our alfalfa extract. It seems now that hydrolysis does destroy growth factors which can be re-introduced by the addition of heat-treated alfalfa. The whole problem of the nitrogen requirements is being investigated more thoroughly in this laboratory and will be reported elsewhere, but it can be stated from experiments with amino acids that the theory of the "peptide requirement" of Tetrahymena is no longer tenable. Kline (1943) came to the same conclusion.

On the basis of our experiments we can say that thiamine is no longer to be regarded as a "growth factor", as defined by Lwoff (1936–37), for *Tetrahymena geleii*. In the presence of adequate amounts of a certain substance or substances of unknown chemical nature, which we have called "factor S," thiamine is synthesized in sufficient quantity to insure rapid and heavy growth. We offer the suggestion that the great size differences noted in declining cultures with and without added thiamine is related to an ultimate depletion of "factor S," and hence thiamine, in the completely dethiaminized media.

It is apparent from a comparison of generation times and population densities that gelatin based media are as good or better than those based on casein. This is only true, however, when gelatin is supplemented with tryptophane. In view of the ease with which gelatin can be handled it is to be preferred to casein.

Summary

1. *Tetrahymena geleii* will grow in serial transplants in completely dethiaminized filtered and diluted casein solution plus salts and riboflavin. Added thiamine has no effect upon the population.

2. Growth rate, population at the end of exponential growth and maximum yield are greatly increased when dethiaminized alfalfa extract is added.

3. No growth occurs in completely hydrolysed casein even with added thiamine.

4. Good growth occurs in casein hydrolysate plus dethiaminized alfalfa extract. Heat and alkali treatment of the hydrolysate or the addition of thiamine have no significant effect.

5. Whole gelatin and dethiaminized whole gelatin support transplantable growth. The addition of thiamine has no effect during logarithmic growth but causes an increase in maximum yield.

6. The addition of dethiaminized alfalfa extract to gelatin and to dethiaminized gelatin increases significantly the reproductive rate, population at the end of logarithmic growth and the maximum yield.

7. Gelatin hydrolysate and dethiaminized gelatin hydrolysate support growth only when alfalfa extract is added. The addition of thiamine has no significant effect.

8. Ciliates growing in gelatin or heated gelatin alone become extremely small and sluggish after about the eighth day. They remain viable up to 30 days, however. The addition of thiamine causes the ciliates to become very large in old cultures. The addition of alfalfa extract produces ciliates intermediate in size.

9. The conclusion is reached that casein and gelatin possesses small amounts of "factor S" which makes it possible for *Tetrahymena geleii* to synthesize thiamine. "Factor S" from alfalfa extract, together with "factors I and II" (Dewey, 1944) added to casein or gelatin, produce rapid and heavy growth. Thiamine is not a "growth factor" for *T. geleii* (strain W).

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X-RAYS AND THE REPRODUCTIVE CYCLE IN RING-NECKED PHEASANTS [1]

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While engaged in a study of the effect of visible radiation on the reproductive cycle in pheasants, we found an opportunity to study the effect on the reproductive cycle of x-raying the heads of pheasants.

Normal breeding in pheasants covers the period from the middle of April to about the middle of July in the latitude of Schenectady, New York. After the last eggs are laid, the gonads atrophy and enter a resting period until the next spring when the cycle is repeated. The birds have a single definite reproductive period, are hardy and fairly easily handled, and therefore make desirable experimental material.

In an attempt to secure stimulation of the pituitary gland with acceleration of the reproductive period, the heads of four female pheasants were radiated with 50 and 75 r. of x-rays and of two females with 225 and 425 r. An equal number of males were similarly radiated. The x-ray tube was operated at 200 K.V., the rays being passed through 0.5 mm. copper filter.[2] All other parts of the body were protected by lead-impregnated fabric. The groups receiving 50 and 75 r. were given the total radiation on December 12, 1938, while those receiving the larger doses were given 25 r. on the same date and the remainder on February 1, 1939. Two pairs of pheasants without radiation served as controls. All birds were the same age, of the same strain, and had been raised in the same flock. Each pair of birds was held in a separate pen at the New York State Research Center, Delmar, New York, until two weeks after laying ceased. All were fed the normal breeding ration as used on the State Game Farms.

TABLE I

Dose in roentgens	Egg laying began	Egg laying ceased	Days of laying period	Average eggs per hen	Average eggs per hen per day during laying period
0	Apr. 13	July 2	50	35.5	0.71
50	Apr. 15	May 24	39	13	0.333
75	Apr. 17	May 21	34	11	0.324
225	Apr. 21	May 15	24	8	0.333
425	Apr. 23	May 8	15	6	0.40

[1] Supported in part by a grant-in-aid from the Society of Sigma Xi.

[2] The radiation was made possible through the cooperation of Dr. Albert Lenz, Schenectady, New York.

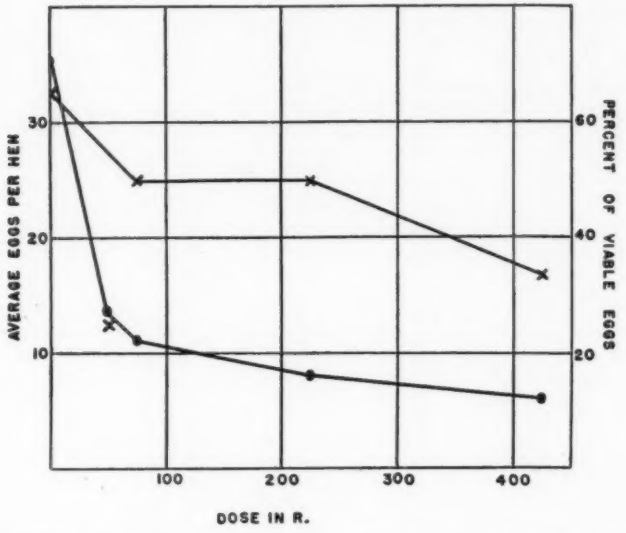

FIGURE 1. Relation between average eggs per hen and percentage of variable eggs and dose of x-rays. Solid circles, average eggs per hen; crosses, percentage of viable eggs.

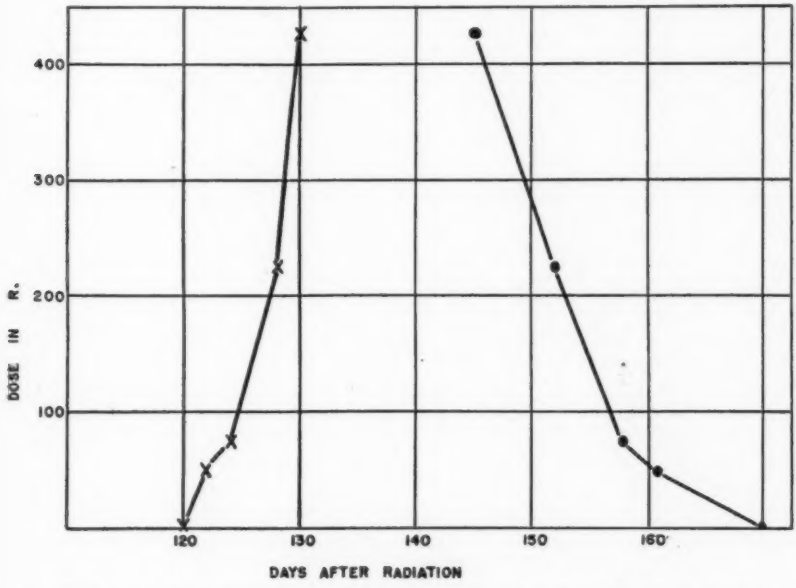

FIGURE 2. Relation between onset and cessation of egg laying and dose of x-rays.

During the 181 days of the experiment no superficial effects of the radiation appeared. Epidermatitis, loss of feathers or other signs indicating localized effects were absent. The weight of the birds fluctuated, but no more than to be expected in any normal group during the breeding season.

The summary of the effects of radiation on reproduction is given in Table I and Figures 1, 2, and 3.

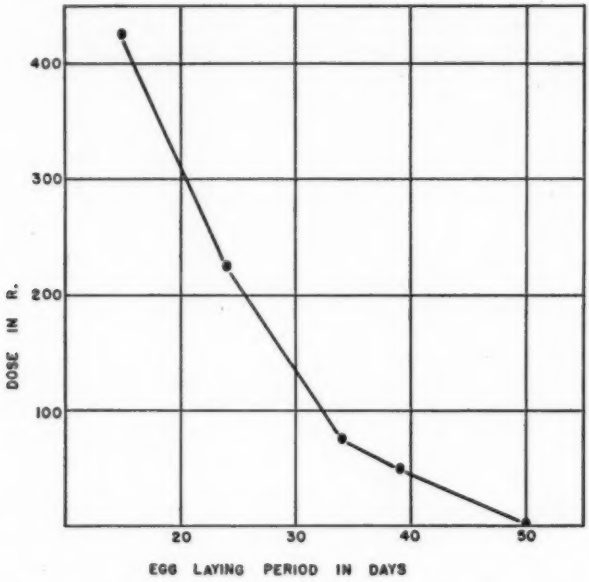

FIGURE 3. Relation between egg laying period and dose of x-rays.

It will be noted (Fig. 1) that the average number of eggs laid per hen varies with the dosage from a high of 35.5 eggs for the controls to 6 eggs for those receiving 425 r.

It will be noted also, Table I and Figure 2, that the time from the beginning of the experiment to the laying of the first egg increases and that the time to cessation of egg laying decreases with the dosage. Thus the laying period varies (Fig 3) from 50 days for the controls to 15 days for birds receiving the maximum radiation. Therefore, the reduced yield of experimental birds must be in part due to their shortened laying period. That this is not the only factor involved is shown in comparing the yield and duration of egg laying of the controls and the birds given 50 r. units of radiation. The ratio of the egg laying periods is 50 : 39 or approximately 1.3 : 1 while the ratio of eggs is 35.5 : 13 or approximately 2.7 : 1. In other words, the controls laid more than twice as many eggs as would be expected if length of laying period were the only factor involved. The rates of egg laying during the active period were calculated and are given in column 6, Table I. It

will be seen that rate of laying is essentially the same for all experimental groups, but is less than half that for the control birds.

Although heads only were radiated and the first eggs were laid at least two months later, the viability of the eggs seemed to be affected. A measure of viability was taken as the percentage of eggs pipped or hatched on incubation. It will be seen (Fig. 1, B) that viability decreased from 65 per cent in the controls to 33 per cent in the group receiving 425 r. An exception is found in the group receiving 50 r. where only 15 per cent of eggs were viable, but this low value was due to one hen, all of whose eggs but two were either infertile or had dead germs. Consequently, although the data for that dosage are given it is not considered reliable.

The simplest assumption is that x-rays in the amounts given decreased the amount of pituitary hormone by damaging the cells or causing a prolonged partial inhibition of function. The decrease in egg production, rate of laying, and length of laying period would be manifestations resulting from pituitary disturbance.

Summary

Pheasants given 50, 75, 225 and 425 r. of x-rays applied to the head region show disturbances in their reproductive cycle by decreased egg production, decreased length of laying period, and possibly decreased viability of eggs laid, the amount of decrease being related to the amount of radiation. The rate of egg laying is decreased in the experimental animals but is not correlated with the dose of x-rays over the range studied.

THE EFFECTS OF POTASSIUM CYANIDE, POTASSIUM ARSENITE, AND ETHYL URETHANE ON RESPIRATION IN PELOMYXA CAROLINENSIS[1]

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Introduction

It has been known for some time that cyanide and carbon monoxide inhibit the normal processes of oxidation in living cells. The effect of cyanide on respiration has been studied both on cells of metazoan tissues and on some of the Protozoa. Lund (1918), Gerard and Hyman (1931), and Shoup and Boykin (1931) all found that respiration in Paramecium is not inhibited by cyanide. Lwoff (1934) found that the respiration of *Glaucoma piriformis* in peptone solution was at first reduced as much as 80 per cent by KCN, but later returned to normal or nearly so. Peters (1929) found that M/500 KCN did not inhibit the respiration of *Colpidium colpoda*. Pitts (1932) found that the respiration of *Colpidium campylum* was slightly reduced by cyanide but that this effect was only temporary. Hall (1941), using an improved technique to avoid the loss of cyanide from the test solution, definitely confirmed the fact that *Colpidium campylum* is sensitive to cyanide.

So far as is known, there are no published investigations dealing with the mechanism of respiration in amoeboid organisms. *Pelomyxa carolinensis* Wilson (*Chaos chaos* Schaeffer), a multinucleate rhizopod, is favorable for physiological studies because it is relatively large and can easily be grown in the laboratory.

Material and Methods

The specimens of *Pelomyxa carolinensis* used in these experiments were of the same strain as those used by Belda (1942) and Pace and Belda (1944). They were grown in Hahnert (1932) solution and were fed by adding paramecia to the cultures. Prior to each experiment, however, the pelomyxae were kept for about a week in a culture solution buffered to maintain a hydrogen-ion concentration of pH 6.8 (Pace and Belda, 1944, Table I). Portions of a centrifuged culture of *Paramecium caudatum* were added every second or third day. The pelomyxae grew well under these conditions and usually contained numerous food vacuoles.

The rate of oxygen consumption was measured by means of a Barcroft-Warburg apparatus. Preliminary tests (Pace and Belda, 1944) had shown that there was no measurable difference in the rate of oxygen consumption between pelomyxae tested in flasks which contained 100, 200, or 300 organisms. In the present series of experiments usually 200 specimens were put into each flask; in a few cases 150 or 300 specimens were used.

[1] With the support of a grant from the American Philosophical Society.

A typical experiment was carried out in the following manner: a 0.4 ml. portion of 10 per cent KOH was put into the inset and a 0.3 ml. portion of 3 N HCl into the onset of 3 of the flasks. These 3 flasks were used as controls. Pelomyxae of uniform size were removed from the buffered culture medium with a capillary pipette under a binocular dissecting microscope and washed in 3 separate portions of fresh sterile culture medium. A 5 ml. portion of sterile culture medium containing the proper number of pelomyxae was then put into each of the 3 Warburg flasks.

A 0.4 ml. portion of a KOH–KCN absorption solution [2] was put into the inset of the 3 remaining flasks and a 0.3 ml. portion of 3 N HCl was put into the onset. Pelomyxae were removed from the buffered culture medium and washed in 3 separate portions of fresh culture medium plus either potassium cyanide, potassium arsenite, or ethyl urethane.

The Barcroft-Warburg apparatus included a total of 7 manometers and flasks. Of these, 6 were prepared as above. A 5 ml. portion of sterile culture solution without pelomyxae was put into the remaining flask which was used as a thermobarometer.

The water bath of the apparatus was kept at 25° ± 0.05° C. The shaking mechanism was operated at the rate of 124 complete cycles per minute through an amplitude of 3 cm. After the manometers and flasks had been put into place with the stopcocks open, the shaking mechanism was run for one hour in order to equalize the temperature of the flasks with that of the water bath. All stopcocks were then closed, and manometer readings were recorded at intervals of one hour.

Results

I. The effect of potassium cyanide on respiration.

In order to ascertain the possible effects of cyanide on the structure and activity of Pelomyxa, several dozen specimens were put into Columbia dishes containing buffered culture solution plus different concentrations of KCN. The specimens were observed carefully under the microscope and compared with other specimens kept in culture solution without KCN.

Practically all the food vacuoles disappear in pelomyxae kept for twelve hours or longer in a solution containing 10^{-2} M KCN. In addition there is a reduction in number or size of both the bipyramidal crystals and the cytoplasmic granules, so that the organisms now appear highly transparent. A number of large vacuoles containing clear fluid are produced in the cytoplasm. Large masses of gelated cytoplasm are found occasionally, both in the interior of the organisms and near the tips of the pseudopodia. Only intermittent movement of the plasmasol can be seen. The hyaline layer appears well-defined, and is much thicker than in normal

[2] The KCN and KOH concentrations of the absorption solutions suggested by Krebs (1935) vary with the KCN concentration of the experimental culture fluid as shown:

Molar concentration of KCN in culture solution	Absorbing solution in inner cup (inset)
10^{-2}	10 ml. 2N KCN + 0.2 ml. N KOH
10^{-3}	10 ml. N KCN + 1.0 ml. N KOH
10^{-4}	5 ml. N KCN + 5.0 ml. N KOH
10^{-5}	1 ml. N KCN + 10.0 ml. N KOH

specimens. The surface of the pelomyxae is covered with small protuberances.

After 24 hours in the solution, the pelomyxae have long, thread-like pseudopodia. Additional clear vacuoles make their appearance and movement of the cytoplasm practically ceases. The plasmagel layer appears to be very thin and it is difficult to handle the organisms without breaking the outer protoplasmic layers. If the outer layer is ruptured no new membrane is formed in the region of rupture, and the cytoplasm flows out into the surrounding culture medium. In lower concentrations of KCN, namely, 10^{-3}, 10^{-4}, and 10^{-5} M, similar effects occur, but in progressively less degree.

When KCN was added to the buffered culture solution in the higher concentrations used (10^{-2} and 10^{-3} M), the hydrogen-ion concentration was reduced. HCl was added to restore the hydrogen-ion concentration to the value of pH 6.8.

TABLE I

The effect of potassium cyanide on oxygen consumption in *Pelomyxa carolinensis*. Temperature 25° C.; hydrogen-ion concentration, pH 6.8. In most of the tests, 200 pelomyxae were used in each flask; in a few tests, 150 and 300 were used. Average volume of one million pelomyxae, 32,000 cubic millimeters.

Molar concentration of KCN	Number of tests	Duration of tests	Average O_2 consumption in $mm.^3$ per hour per million organisms	Average O_2 consumption in $mm.^3$ per hour per $mm.^3$ cell substance	Per cent inhibition
0 (Control)	8	3 to 6 hours	9045±595	0.282±0.018	65.4
10^{-5}	8		3132±387	0.098±0.012	
0 (Control)	9	3 to 5 hours	8962±641	0.280±0.020	63.1
10^{-4}	9		3220±302	0.100±0.009	
0 (Control)	7	4 to 5 hours	8718±548	0.272±0.017	67.5
10^{-3}	7		2840±345	0.089±0.010	
0 (Control)	8	3 to 4 hours	9478±567	0.296±0.018	69.1
10^{-2}	8		2930±248	0.092±0.008	

The results of the experiments with cyanide are shown in Table I. This table shows that there is a reduction of 63 to 69 per cent in the rate of oxygen consumption in Pelomyxa when KCN is present in the culture solution in concentrations of 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M. The highest concentration of KCN, however, produced only slightly greater inhibition than the lowest. These results indicate that cellular oxidation in Pelomyxa is regulated principally, but not entirely, by the cytochrome-cytochrome oxidase system.

II. The effect of potassium arsenite on respiration

Lwoff (1934) found that respiration in *Glaucoma piriformis* is only slightly reduced, and under certain conditions actually increased in the presence of KCN, and concluded that some respiratory mechanism other than that of cytochrome-cytochrome oxidase must be present in this organism. In an attempt to ascertain whether this mechanism might involve glutathione, he tested specimens of Glaucoma

with sodium arsenite. This reagent inhibits the activity of glutathione and other compounds containing –SH groups, but presumably does not affect cytochrome oxidase. Lwoff found that in solutions of 5.26×10^{-4} M and 8.7×10^{-4} M sodium arsenite, respiration in Glaucoma was reduced, respectively, by 75–80 and 90 per cent.

Experiments were carried out with Pelomyxa as above, except that solutions of potassium arsenite instead of potassium cyanide were used. Potassium arsenite was added to buffered culture solution to produce concentrations of 5×10^{-3}, 10^{-3}, 5×10^{-4}, and 10^{-4} M. Additional tests were made with higher concentrations of potassium arsenite, but the organisms were killed in less than 1 hour so that no measurements of changes in the rate of oxygen consumption could be made. The results of the experiments are presented in Table II.

TABLE II

The effect of potassium arsenite on oxygen consumption in *Pelomyxa carolinensis*. Temperature, 25° C.; hydrogen-ion concentration, pH 6.8. Two hundred pelomyxae were used in each flask. Average volume of one million pelomyxae, 34,500 cubic millimeters.

Molar concentration of $KAsO_2$	Number of tests	Duration of tests	Average O_2 consumption in $mm.^3$ per hour per million organisms	Average O_2 consumption in $mm.^3$ per hour per $mm.^3$ cell substance	Per cent inhibition
0 (Control)	6	4 to 5 hours	10,365±467	0.300±0.013	16.3
10^{-4}	7		8,680±525	0.252±0.015	
0 (Control)	6	5 hours	10,240±1,140	0.297±0.033	22.0
5×10^{-4}	6		8,000±467	0.231±0.013	
0 (Control)	7	3 hours	9,894±500	0.287±0.014	29.0
10^{-3}	6		7,025±594	0.203±0.017	
0 (Control)	6	3 to 5 hours	9,831±510	0.285±0.015	34.9
5×10^{-3}	6		6,403±506	0.185±0.015	

This table shows that there is a progressive decrease in oxygen consumption with increasing concentrations of $KAsO_2$. The maximum decrease obtained with the highest concentration of $KAsO_2$ which was not lethal to the pelomyxae was 35 per cent.

III. The effect of ethyl urethane on respiration

Some of the mechanisms of cellular oxidation involve the dehydrogenases. These may be inhibited by the urethanes. Lwoff (1934) found that respiration in *Glaucoma piriformis* was reduced by methyl, ethyl, and propyl urethanes. With 1.66 per cent and 2 per cent ethyl urethane the reduction in oxygen consumption was, respectively, 44 and 57–61 per cent.

Experiments were carried out with *Pelomyxa carolinensis* as above, using ethyl urethane in amounts which yielded concentrations of 0.11 M (1 per cent) and 0.17

M (1.5 per cent) after mixture with culture solution. The results are presented in Table III.

This table shows that with 0.11 M and 0.17 M ethyl urethane the rate of respiration in Pelomyxa decreased, respectively, 35.7 and 65.1 per cent.

TABLE III

The effect of ethyl urethane on oxygen consumption in *Pelomyxa carolinensis*. Temperature, 25° C.; hydrogen-ion concentration, pH 6.8. Two hundred pelomyxae were put into each manometer flask. Average volume of one million organisms, 31,200 cubic millimeters. Duration of each test, 3 hours.

Molar concentration of ethyl urethane	Number of tests	Average O_2 consumption in $mm.^3$ per hour per million organisms	Average O_2 consumption in $mm.^3$ per hour per $mm.^3$ cell substance	Per cent inhibition
0 (Control)	5	10,670±1,240	0.341±0.040	
0.11	6	6,860± 710	0.219±0.023	35.7
0 (Control)	7	9,210±1,100	0.295±0.035	
0.17	6	3,220± 470	0.103±0.015	65.1

DISCUSSION

The results obtained by investigators in earlier tests with cyanide may be erroneous because of rapid loss of HCN from the test solutions. Failure to obtain inhibition of respiration in Paramecium with cyanide has led to the conclusion that the cytochrome-cytochrome oxidase system is not involved in respiration in this organism. However, since Saito and Tamiya (1937) have reported the presence of cytochrome a and c in Paramecium, additional tests, more accurately controlled, should be made.

The fact that Pitts (1932) and Lwoff (1934) obtained only temporary inhibition of respiration in ciliates with KCN, and that Lwoff (1934) obtained inhibition with KCN in peptone solution but not in glucose-Ringer solution, may have been due to loss of cyanide from the test solutions.

The decreased rate of respiration in *Pelomyxa carolinensis* induced by potassium cyanide was maintained as long as the specimens were kept in the solutions, whereas the inhibition brought about by potassium arsenite was only temporary. After eight or nine hours in $KAsO_2$ the rate of respiration had returned nearly to normal; after 15 to 18 hours it was completely normal. These results may indicate that after the supposed inhibition of the glutathione mechanism in Pelomyxa some other respiratory mechanism may begin to function. On the other hand, it may be that there has occurred a gradual conversion of arsenite in the test solution to arsenate.

There is some evidence in the results obtained by Szent-Györgyi and Banga (1933), Korr (1935), and Cohen and Gerard (1937), that arsenite may inhibit the activity not only of glutathione but also of dehydrogenases. If this be true, the results with Pelomyxa indicate that arsenite inhibits the action of dehydrogenases much less than does urethane.

The degree of inhibition of respiration by ethyl urethane in Pelomyxa is approximately equal to that in Glaucoma (Lwoff, 1934), but in approximately equal concentrations of arsenite, respiration in Pelomyxa is inhibited initially by 29 per cent, compared to 75–80 per cent in Glaucoma. If, as supposed, arsenites inhibit the activity of glutathione but not that of respiratory enzymes, it appears that glutathione is much less important in the respiration of Pelomyxa than of Glaucoma.

These results indicate that the respiratory mechanism of *Pelomyxa carolinensis* differs considerably from that of some of the Ciliata. Whether or not the mechanism of respiration of *Pelomyxa carolinensis* resembles that of other free-living Rhizopoda must await further investigation.

Summary

1. In 10^{-5} M KCN respiration in *Pelomyxa carolinensis* is inhibited by 63 per cent. In much higher concentrations of KCN, up to 10^{-2} M, only slightly greater inhibition occurs.

2. Pelomyxae which have been exposed to potassium cyanide (10^{-5} to 10^{-2} M), for 12 to 24 hours, show many changes in protoplasmic structure.

3. In 5×10^{-3} M potassium arsenite the maximum inhibition of respiration in *Pelomyxa carolinensis* is 35 per cent; this effect, however, is only temporary.

4. In 0.17 M (1.5 per cent) ethyl urethane the respiration of *Pelomyxa carolinensis* is inhibited by 65 per cent.

5. Respiration in *Pelomyxa carolinensis* appears to occur chiefly through a cytochrome-cytochrome oxidase system, and partly through a mechanism involving glutathione.

6. The respiratory mechanism of *Pelomyxa carolinensis*, a rhizopod, differs considerably from that of a number of the ciliates.

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THE EXTERNAL MORPHOLOGY OF THE THIRD AND FOURTH ZOEAL STAGES OF THE BLUE CRAB, CALLINECTES SAPIDUS RATHBUN [1]

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For the past two years, workers at the Virginia Fisheries Laboratory, Williamsburg, have been attempting to rear larvae of the commercially important blue crab from the egg through all zoeal stages. In 1941 Dr. Margaret S. Lochhead worked out a successful method of hatching the eggs (Lochhead, Lochhead and Newcombe, 1942) and reared the larvae to the "second zoea" stage. During the summers of 1942 and 1943 this work was continued by Mrs. Mildred Sandoz and Miss Rosalie Rogers, who succeeded in rearing a number of individuals to the "third zoea" stage. The anatomy of the first and second zoeal stages was described in detail by Hopkins (1943). Churchill (1942) described five zoeal stages found in plankton tows at the mouth of Chesapeake Bay. Churchill's first and second zoeae seem to be identical with those reared from blue crab eggs at the Virginia Fisheries Laboratory, but his third zoea is markedly different from the third zoea reared at this laboratory, as reported by Sandoz and Hopkins (1944).

It is now realized, by the agencies concerned with regulation of the crab fishery in Chesapeake Bay, that a more detailed knowledge of the biology of the crab is necessary. Studies of the numbers and seasonal and geographic distribution of larvae in all stages are important means of locating the breeding grounds and determining the length of the larval period, the migrations of the larvae, the percentage of survival under natural conditions, etc. Obviously these studies will be worthless unless the blue crab larvae are correctly identified, and distinguished from the other species of the same family (Portunidae) found in this region. The following paragraphs attempt to give an accurate and detailed description of all features which may be of importance in separating larvae of different species.

A number of individuals in the "fourth zoea" stage have been found in plankton tows made by the Virginia Fisheries Laboratory in the mouth of the Bay near Cape Henry. These are very similar to the blue crab "third zoea" raised in the laboratory and found in plankton, but quite different from Churchill's "fourth zoea" (which was also found in our tows).

The first and second zoeal stages have been restudied, and a few minor corrections of my 1943 description seem necessary. In the first zoea, there are six setae on the endopodite of the first maxilla, two in one group and four in the other. In the second zoea, there are normally three apical setae on the scaphognathite of the second maxilla, although only two can be seen in some specimens. The shorter seta on the fourth segment of the endopodite of the first maxilliped is usually longer than indicated in my 1943 report.

[1] Joint contribution from the Virginia Fisheries Laboratory of the College of William and Mary and Commission of Fisheries (Number 20): and from the A. and M. College of Texas.

The Third Zoea

Three specimens reared in the laboratory and six found in plankton tows (Cape Henry, August 14, 1941) were dissected and mounted in glycerine. In addition, a number of specimens from plankton and one specimen reared in the laboratory were mounted and studied entire, and still others were studied in formalin without mounting. Even after the most detailed study, no difference could be found between laboratory-reared and plankton specimens, except that one laboratory-reared specimen seemed to have moulted precociously (the telson lacked the fourth or inner pair of setae, the second maxilla was of the second zoea type, and the size was below normal, although the maxillipeds each bore eight swimming hairs).

The total length of the body is difficult to measure accurately because of the bent position of most specimens, but varies between 1.40 and 1.65 mm., measured from front of carapace between eyes to tips of telson. The carapace is exactly as in the second zoea except for larger size and the presence of a single (occasionally two) seta with setules on the posterior edge of the carapace. The dorsal spine is 0.40 to 0.50 mm. long, the lateral spines are 0.09 to 0.10 mm., and the rostrum is 0.33 to 0.36 mm. (measured from lower edges of bases of eyestalks). The eyes have very short stalks and are 0.21 to 0.26 mm. in diameter (Figs. 1 and 2).

The antennule is unchanged from its form in the second zoea except that the setae or aesthetes are less uniform in width; the largest aesthete is about twice as wide as the second, the second is almost twice as wide as the third, and the third is about twice as wide as the fourth; there is also a very short bristle or seta which usually cannot be seen. The peduncle of the antennule is 0.12 to 0.15 mm. long and the length of the longest aesthete is about 0.20 mm. (Fig. 3).

The antenna is 0.30 to 0.35 mm. long. The spinous process bears 12 to 18 hooklike spines on each side. The exopodite is about 0.01 mm. long and bears two setae of unequal length, the longer 0.03 to 0.04 mm. long. There is a very slight ridge or bulge on the antenna near the exopodite which represents the first rudiment of the endopodite.

The labrum bears a distinct chromatophore. The mandible is 0.12 to 0.19 mm. long and 0.08 to 0.11 mm. wide, and bears a very large chromatophore usually divided into three parts.

The maxillule or first maxilla is 0.16 to 0.22 mm. long, from base to end of endopodite. The outer edge of the basipodite bears a seta about 0.04 mm. long. The distal (basal) endite of the protopodite bears eight setae and the proximal (coxal) endite bears six or seven setae. The endopodite is two-segmented; the

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Plate I

The third zoeal stage of *Callinectes sapidus*. All figures are camera lucida drawings Scale line A represents 0.5 mm. in Figures 1 and 2; scale line B represents 0.2 mm. in Figures 3–7.

Figure 1. Third zoea reared from egg in laboratory, slightly flattened under cover glass.
Figure 2. Third zoea from plankton tow, Cape Henry.
Figure 3. Antennule, third zoea from plankton.
Figure 4. First maxilla, third zoea reared in laboratory.
Figure 5. Second maxilla, third zoea from plankton.
Figure 6. Endopodite of first maxilliped, third zoea reared in laboratory.
Figure 7. Endopodite of second maxilliped, third zoea reared in laboratory.

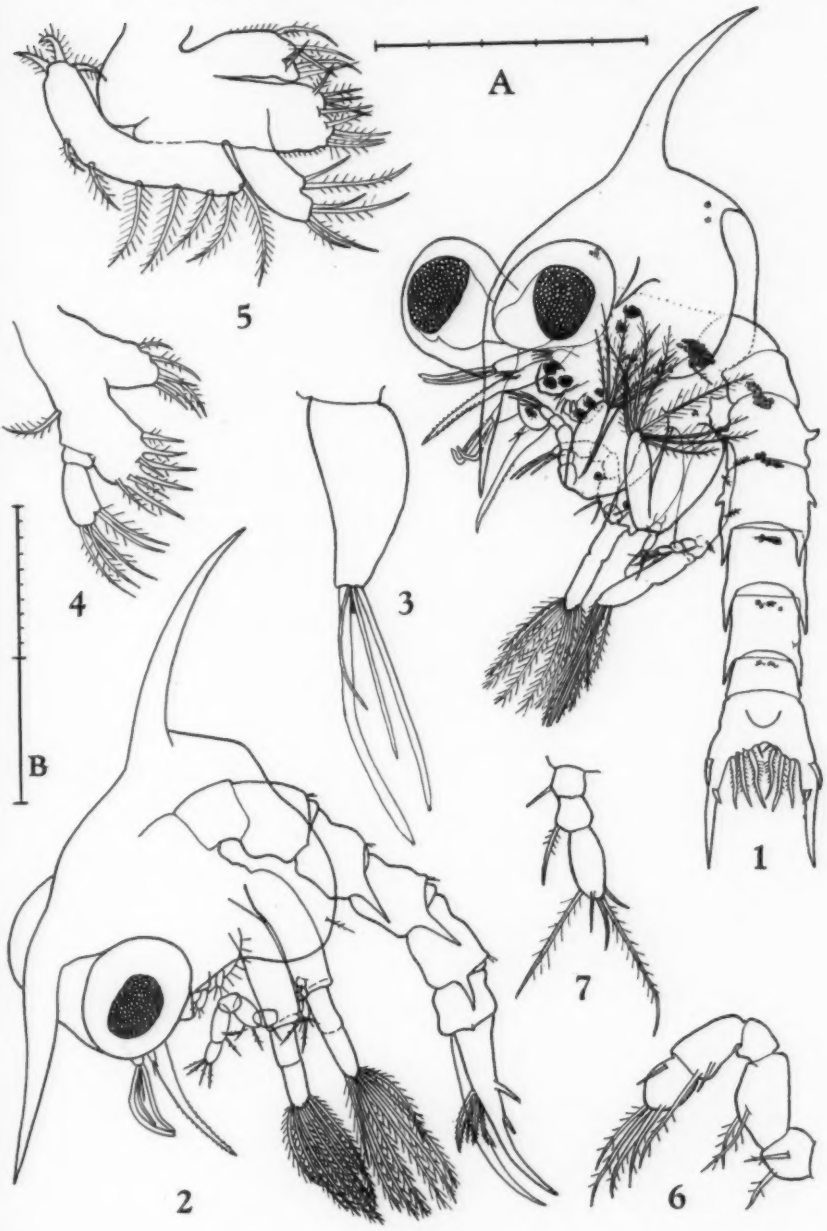

PLATE I

distal segment is bifurcated and bears two groups of setae, four in one group and two in the other. The setae on the basal and coxal endites are 0.03 to 0.06 mm. long and those on the endopodite are 0.04 to 0.08 mm. long; all setae on the first maxilla have setules (Fig. 4).

The second maxilla seems to be subject to considerable variation in size and number of setae. The length of the second maxilla varies from 0.12 to 0.17 mm., measured from the base to the tip of the endopodite. The scaphognathite measures from 0.13 to 0.16 mm. through its longest dimension, and bears seven or eight setae along the distal part of the outer margin plus three to five apical setae around the proximal tip, making a total of ten to twelve setae. The endopodite has a single segment with bilobed tip bearing six setae in two groups, two in one group and four in the other. The basal and coxal endites are both bilobed; the distal (basal) endite bears nine setae and the proximal (coxal) one bears six or seven setae. All setae on the second maxilla bear setules and there are also setules around the margins of the endites (Fig. 5).

The basipodite of the first maxilliped is 0.20 to 0.27 mm. long; its posterior side bears ten setae with setules, and there is a very distinct chromatophore near its distal end. The endopodite is 0.18 to 0.23 mm. long, and has five segments; the first (proximal) segment bears two setae, the next bears two, the third has none, the fourth bears two, and the fifth bears five setae, four terminal and one short lateral seta (Fig. 6). The exopodite is divided into two segments; its length is 0.16 to 0.18 mm.; the eight terminal setae or "swimming hairs" are of unequal lengths, varying from 0.13 to 0.23 mm.

The basipodite of the second maxilliped is 0.20 to 0.24 mm. long and bears four setae with setules near its posterior margin; there is no chromatophore in the basipodite, but there is a rather small chromatophore in the coxopodite. The endopodite is 0.06 to 0.09 mm. long and consists of three segments; the first (proximal) and second segment each bear one seta and the third bears five unequal terminal setae (Fig. 7). The exopodite is 0.18 to 0.23 mm. long, is divided into two segments, and bears eight terminal setae or "swimming hairs" of unequal lengths, 0.15 to 0.27 mm.

The abdomen is 1.05 to 1.20 mm. long and has six segments, not counting the telson. The lateral spines or hooks on the second and third segments are exactly as in the second zoea. The third, fourth, and fifth segments have lateral spines projecting posteriad from the posterior margin of each segment. The second, third, fourth, and fifth segments each have a pair of dorsal setae projecting from the posterior margin, but there is no sign of a dorsal spine on any segment. The first

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PLATE II

The fourth zoeal stage of *Callinectes sapidus*. All figures are camera lucida drawings. Scale line A represents 0.5 mm. in Figure 8; scale line B represents 0.2 mm. in Figures 9–14.

FIGURE 8. Fourth zoea from plankton tow, Cape Henry.
FIGURE 9. Antennule.
FIGURE 10. Antenna.
FIGURE 11. First maxilla.
FIGURE 12. Second maxilla.
FIGURE 13. Endopodite of first maxilliped.
FIGURE 14. Endopodite of second maxilliped.

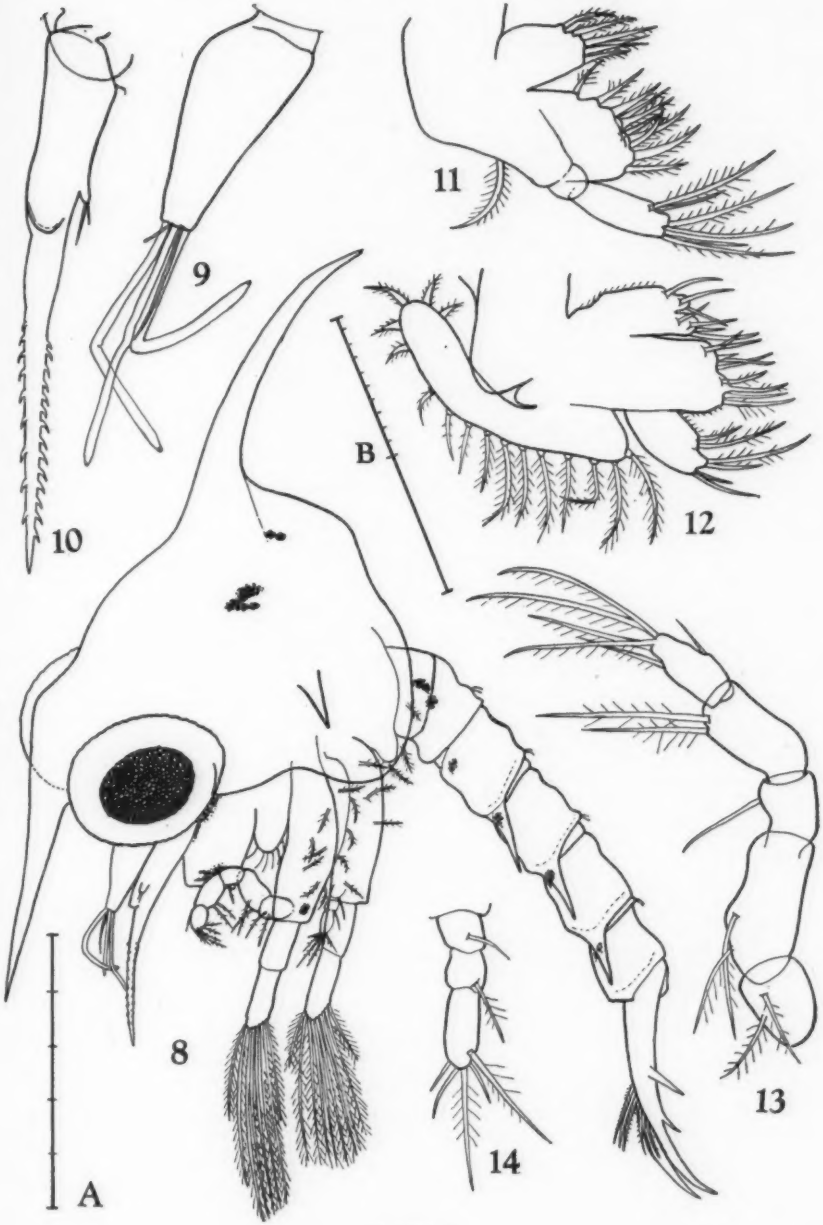

PLATE II

abdominal segment bears a large median chromatophore; each of the other segments bears a chromatophore lying across the extreme anterior end near the ventral surface. Except for slightly larger size, the telson is unchanged from its condition in the second zoea; that is, the dorsal side of each furcus bears a large spine directed dorsolaterally, and a smaller spine, farther back, directed dorsally, and the inner side of each furcus bears close to its base a small spine without setules in addition to the original three setae with setules (Fig. 1).

The chromatophores probably cannot be described accurately without using living specimens, but the following chromatophores have been seen in the best preserved specimens: (1) one chromatophore in the front of the head, between the eyes; (2) one chromatophore dorsal to the anterior part of the alimentary canal; (3) a pair of large chromatophores dorsal to the gut in the posterior part of the cephalothorax; (4) a pair of chromatophores just ventral to the anterior part of the alimentary canal; (5) a small chromatophore, not always visible, below the base of the dorsal carapacial spine; (6) a large chromatophore in the center of the first abdominal segment; (7) chromatophores in the ventral anterior margin of the third, fourth, fifth, and sixth abdominal segments; (8) a large chromatophore in the labrum; (9) a large chromatophore in each mandible; (10) a chromatophore in the distal end of the basipodite of each first maxilliped; (11) a chromatophore in the coxopodite of each second maxilliped.

The Fourth Zoea

Several specimens which are tentatively identified as the "fourth zoea" of the blue crab were found in surface plankton tows near Cape Henry, August 14, 1941. Five of these specimens have been studied in formalin solution, and two have been dissected for more detailed study of appendages. These specimens are identical with the third zoea of the blue crab except for larger size, a better developed endopodite bud on the antenna, additional setae on the maxillae and maxillipeds, and more setae on the posterior edge of the carapace. Identification of this zoea should, of course, be checked by comparison with laboratory-reared fourth zoeae when these can be obtained, but, in the meantime, I have little or no doubt that this is actually the fourth zoea of the blue crab.

The length of the body, from midway between eyes to tips of the telson, is 1.75 to 1.95 mm. The dorsal spine on the carapace is 0.54 to 0.60 mm. long and the lateral spines are about 0.12 mm. long. The length of the rostrum is from 0.45 to 0.50 mm. The eyes are about 0.35 mm. in diameter. The posterior edge of the carapace on each side bears three large setae with setules (Fig. 8).

The antennule is almost exactly as in the third zoea; the peduncle is 0.16 mm. long and the longest aesthete is 0.20 mm. (Fig. 9). The antenna is from 0.35 to 0.42 mm. long and is unchanged from the third zoeal stage except that the bud of the endopodite is now very distinct (Fig. 10); it is noteworthy that the exopodite is still of exactly the same form as in the first zoea. The labrum and mandible are as in the third zoea except for slightly larger size. The maxillule or first maxilla differs from the third zoeal stage only in slightly larger size and in having nine or ten setae on the distal (basal) endite where the third zoea had eight (Fig. 11). The scaphognathite of the second maxilla bears about sixteen setae, with only a slight gap between the setae of the outer margin and the apical setae, which are now similar

in form. The basal and coxal endites of the second maxilla are as in the third zoea except that the distal (basal) endite bears nine or ten setae and the proximal (coxal) one has seven (Fig. 12).

The first maxilliped bears eight setae or "swimming hairs" on the exopodite. The endopodite is five-segmented; the first segment bears two setae, the second bears two, the third bears one (lacking in some specimens), the fourth has two, and the terminal segment bears six setae of which four are terminal (Fig. 13). The basipodite of the first maxilliped bears ten setae, located mostly along its posterior edge. The basipodite is 0.28 mm. long, the exopodite is 0.23 to 0.26 mm., and the endopodite is 0.25 to 0.31 mm. The longest seta on the exopodite is 0.32 mm. long. As in previous stages, there is a chromatophore in the distal end of the basipodite (Fig. 8).

The second maxilliped bears ten terminal setae on the exopodite. The endopodite is three-segmented; the first and second segments each bear one seta and the terminal segment has five setae (Fig. 14). The basipodite bears four setae and contains no chromatophore, but the coxopodite does have a chromatophore. The basipodite is 0.30 mm. long, the exopodite 0.24 mm., and the endopodite 0.09 to 0.11 mm. The longest seta on the exopodite is 0.31 to 0.34 mm. long.

The abdomen, including the telson, is identical in every detail with the abdomen of the third zoea, except for larger size.

Discussion

Churchill (1941, 1942) has described five zoeal stages which he assigned to *Callinectes sapidus*. His first zoea and second zoea do belong to this species; they agree with our laboratory-reared zoeae in every detail except that Churchill apparently overlooked the little dorsal spine on each furcus of the telson, which is present in all blue crab zoeae from the first. However, Churchill's third and fourth zoeae, and presumably his fifth zoea also, belong to a different species of crab and have nothing to do with *C. sapidus*. Churchill's third zoea is slightly larger than our laboratory-reared blue crab third zoea, but the most striking differences are the presence of prominent dorsal spines on the fifth abdominal segment (lacking in *C. sapidus*) and the much greater length of the antennal exopodite (which in *C. sapidus* is unchanged from the second zoeal stage). Churchill's third zoea has six swimming hairs on the exopodite of the first maxilliped, and seven on the second, while *C. sapidus* has eight on each maxilliped. There are also minor differences, including the number of setae on the carapace, the degree of development of the appendages following the second maxilliped, and perhaps the numbers of setae on the endopodites of the maxillipeds.

I did not find Churchill's third zoea in my plankton tows, but I did find one specimen of his fourth zoea (in a surface tow off Ocean View, Va., Aug. 14, 1941) and can confirm the accuracy of his excellent figure and description of this stage. Churchill's fourth zoea is much more robust than the fourth zoea which I have assigned to *C. sapidus;* the larger size is obvious to the naked eye. In addition to the possession of dorsal spines on the fifth abdominal segment and the elongated antennal exopodite, his species differs from mine by having seven (instead of three) setae on the posterior edge of the carapace, nine swimming hairs (instead of ten) on the exopodite of the second maxilliped, and greater development of the buds of

appendages posterior to the second maxilliped. The distal pair of spines on the furci of the telson are much smaller than the corresponding spines in blue crab zoeae and are on the inner side of the furci rather than the dorsal side as in *C. sapidus*. Both maxillipeds contain chromatophores in the basipodites in Churchill's species, while only the first maxillipeds of *C. sapidus* have chromatophores in the basipodite.

Churchill's description of five zoeal stages which he assigned to *C. sapidus* was based entirely on specimens found in plankton, with the exception of the first zoea. The Virginia Fisheries Laboratory has hatched thousands of blue crab eggs in the laboratory and has reared a few specimens through the second and third zoeal stages, so that the identity of these stages is known beyond question. The identity of the fourth zoea, described and assigned to *C. sapidus* in the present paper, has not been confirmed in this way.

It is certain that Churchill's third zoea does not belong to *C. sapidus;* probably it is a zoeal stage of some other crab of the family Portunidae. The zoeal stages of *Ovalipes ocellatus, Arenaeus cribrarius, Bathynectes superba,* and *Callinectes ornatus* (species which live in or near the mouth of Chesapeake Bay) have never been described, so it is possible that Churchill's species belongs to one of these. It seems probable that his fourth and fifth zoeae belong to the same species as his third zoea, and it is practically certain that they do not belong to *Callinectes sapidus*. The zoeae of several species of *Portunus* described by Lebour (1928) have a long antennal exopodite like Churchill's zoea, but none of them have dorsal spines on the fifth (or any other) abdominal segment.

It seems probable that *C. sapidus* has a fifth zoeal stage which has not yet been seen by anyone.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1944

JULY 18

Melanophore control of the sexual dimorphism of feather pigmentation in the Barred Plymouth Rock. B. H. Willier.

This report deals with the effects of sex-linked genes on the expression of melanophores in the formation of sexual differences in the pigmentation pattern of feathers of the Barred Rock fowl. Melanoblasts from Barred Rock individuals of known sex were introduced into feather germs of the same or of a different breed of fowl, either by grafting them directly into the wing bud of a host embryo or by allowing them to migrate into melanoblast-free wing skin grafts. All possible combinations between melanoblasts of male and female genotypes and the sex of the host and sex genotype of the grafted skin were obtained. A black and white barred pattern was produced in host or graft contour feathers which was invariably in accordance with the sex genotype of the melanoblast introduced. The melanoblasts of the male which are homozygous for two dominant sex-linked genes, barring and silver, and for a dominant autosomal gene, extension, produced a narrow gray-black band and a wide, almost pure white band. Melanoblasts of the female which are homozygous for extension and hemizygous for barring and silver produced a wide black band and a narrow gray-white band. Thus the melanophores of male and female genotypes are provided with different properties for controlling (*a*) the relative width of the light and dark bands, and (*b*) the intensity of their pigmentation. The difference in expression appears to be determined by the number of sex-linked bar genes interacting with the rest of the genotype (mainly with extension and silver). Furthermore, this difference in expression of melanophores of male and female genotypes is manifested independently of sex hormones of the host and of the sex genotype of the feather germs of the grafted skin. It becomes clear, therefore, that sexual dimorphism of barring is a manifestation of differences in expression of male and female melanophores as provided by the number of sex-linked bar genes interacting with the rest of the genotype.

Since melanophores of the same genotype (from same individual), either male or female, produced a barred pattern which varies in quality from feather to feather in the host or grafted skin, it is evident that the individual feather germ has a more or less specific modifying influence on the rhythmic production of pigment by the melanophores. Growth rate appears to be one of the modifying factors since (1) in flight feathers the variations in barred patterns and in growth rate are roughly parallel, and (2) the differences in barred patterns produced in homologous flight feathers of the host and its donor by melanophores of the same genotype appear to be correlated with breed differences in growth rate, being generally higher in the white Leghorn host than in its barred donor. An increase in growth rate is usually correlated with an increase in the relative amount of black pigment in the vane and with a decrease in the distinctness of barring. Other modifying factors acting simultaneously in the feather germ are likewise involved.

The effects of peripheral factors on motor neuron differentiation in the chick embryo. Viktor Hamburger.

If a wing or leg primordium is extripated in a two- or three-day embryo, the spinal ganglia as well as the lateral motor columns of the corresponding parts of the spinal cord become *hypoplastic*. If the central nervous system is overloaded by implantation of a supernumerary limb, a *hyperplastic* effect is observed. The question arises whether the periphery affects the growth of the central nervous system by controlling the mitotic activity or the cellular differentiation. The lateral motor column was selected for an analysis of this problem.

Mitotic counts were made in 20 wing extirpation cases, during the peak of mitotic activity (in most cases on the fifth and sixth day of incubation). A slight depression of the mitotic activity was found on the operated side. However, the observations to be reported presently show that this response is transient and not related to the hypo- or hyperplasia of the lateral motor column.

If cellular proliferation is not affected by wing extirpation or transplantation, then the sum total of all cells of the spinal cord should remain identical on the operated and the unoperated sides of the cord. This expectation was borne out by cell counts of the motor cells, and, separately, non-motor cells in the ventral half of the cord of five older embryos (two cases of wing-bud extirpation, two cases of leg bud extirpation, and one case of wing implantation). In all instances the total cell numbers were strikingly similar on the two sides. In all instances of hypoplasia, moreover, the deficit of large motor neurons was almost exactly compensated by an excess of small non-motor cells; in the case of hyperplasia, a surplus of motor cells was accompanied by a smaller number of non-motor cells. These data give convincing evidence that the proliferative activity of the cord is not permanently impaired by the operation; they leave no doubt but that the peripheral factors control the process of differentiation of small indifferent cells into large motor neurons. An inductive effect is postulated which emanates from a small group of pioneer motor neurons, and which spreads over adjacent indifferent cells, inducing them to differentiate. The newly recruited neurons are added to the lateral motor column and thus increase its inductive capacity. This process of augmentation is not a self-perpetuating mechanism, however, but is under the "remote control" of conditions prevailing at the periphery. It is cut off at the moment when the periphery is saturated with nerve supply. Under experimental conditions, this would happen earlier (hypoplasia) or later (hyperplasia) than under normal conditions.

The superficial gel layer and its role in development. Warren H. Lewis.

Probably every cell and egg and ameboid organism has a superficial gel layer. It exerts continuous contractile tension, a fundamental property of gelled protoplasm. Gel layer and endoplasm are reversible states of the same cytoplasm that readily changes from one state to the other. Local increases and decreases of its contractile tension are responsible for many changes of cell form, extension of nerve axones, cell locomotion, flow of endoplasm, and cleavage of cells and eggs; and during development for the infiltration and interpenetration by migration of individual cells among others of their own type, nerve cells for example, and among others of different types, capillary endothelial spreading, fibroblast infiltrations and myoblast migrations.

The contractile tension of the gel layer over the yolk of the zebra fish egg squeezes endoplasm out of the yolk to form the blastodisc and compresses the yolk globules into polyhedrons. After cleavage this gel layer pulls the blastodisc over the yolk (gastrulation).

Mechanics of invagination. A relative increase of the contractile tensions of the gel layers on one surface of a series of adherent epithelial cells will result in a concave depression (invagination) on the side of the greater tension. Invagination is the resultant of two forces, a distorting one (the contractile tension of the adherent gel layers) and a resisting one (resistance of the cells to distortion). The cells suffer less distortion because of the invagination. In amphibia, an increase in the contraction of the adherent gel layers of the outer surfaces of the blastophore cells is responsible for one phase of blastopore invagination(gastrulation). This contraction also pulls the presumptive endoderm and mesoderm into the walls of the primitive archenteron and the presumptive ectoderm and neural plate towards the blastopore in spite of an opposing contractile tension exerted by the adherent gel layers of the surface cells over the rest of the egg.

The same mechanical principle is involved in the neural tube formation and its subsequent bendings; optic vesicle evagination and invagination to form the optic cup; invaginations of the lens, otic vesicle, nasal pits, and probably in the evaginations of the thyroid, lungs, liver, pancreas, etc.

It also plays a leading and revealing role in the wound healing of eggs.

The contractile tensions exerted by the superficial gel layer of cells thus plays a leading role in early morphogenesis and probably in later stages also, but the role it plays is dependent upon cytoplasmogenesis, cell division, cell growth (increase in protoplasmic mass), cell adhesions, accumulation of intercellular products, etc.

JULY 25

Ferritin and iron metabolism. L. Michaelis.

A considerable amount of iron is stored in mammalian tissues which is not in the form of any iron-porphyrin compound. 60 years ago Schmiedeberg prepared an iron-containing protein from liver, which he designated as ferratin. It was an ill-defined, not easily reproducible substance. In 1935 Lauffberger discovered that a protein containing as much as over 20 per cent of iron can be obtained as a well-crystallized compound in the form of its cadmium salt from horse's spleen and many other organs of various species. It was designated as ferritin. In 1942, S. Granick and L. Michaelis elaborated a method of separating the iron from the protein. This protein was designated as apoferritin. It resembles the globulins and is not, and does not contain, any nucleoprotein. Whereas ferritin, in spite of its high ability to crystallize, is non-homogeneous on ultracentrifugation, apoferritin is a perfectly homogeneous protein of molecular weight 500,000, as determined by Dr. Rothen. This protein is highly antigenetic. The precipitin reaction shows that it is essentially different from all other known proteins, that it is species-specific, but not organic-specific. Ferritin and apoferritin cannot be distinguished by the precipitin test. Ferritin is brown, apoferritin colorless. In spite of the fact that about 20 per cent of its weight is withdrawn from ferritin, in the form of Fe, on converting it to apoferritin (or about 30 per cent of its weight in the form of ferric hydroxide), the crystal form of apoferritin and ferritin are alike even to such an extent that Dr. Fankuchen could find no essential difference in the X-ray diffraction pattern of the two, except for the fact that diffraction lines are stronger in ferritin than in apoferritin.

The method of preparing ferritin depends on the fact that in an aqueous organ extract, on heating at 80° C. (but not higher) most of the proteins are coagulated, but not ferritin. From the filtrate the remaining protein is salted out by 30 per cent ammonium sulfate, the coagulum redissolved in water and $CdSO_4$ is added. Crystallization proceeds rapidly. The crystals are always isotropic, in the cubic system, either octahedra, often twinned octahedra (horse), or cubes, or tetrahedra in other animals. Human organs yield crystals with slightly curved faces, less regularly shaped. Ferritin can be prepared from many mammals, but not so far from cats or deer, or non-mammalian vertebrates, or invertebrates. It is found in spleen, liver, and bone marrow, and to a small extent (and usually with a smaller iron content, resembling apoferritin), in kidney and testes, but not in blood or muscle. When radioactive iron is injected into anemic dogs, the iron can be retraced after a short time as ferritin in the liver, and under certain circumstances also in the spleen.

The iron of ferritin is always in the ferric state, it is paramagnetic and has a magnetic susceptibility of a characteristic magnitude which does not occur in any other normally occurring iron compound of the organism. It corresponds to a magnetic moment, per gram-atom Fe, of 3.8 Bohr magnetons, which according to theory indicates the presence of three unpaired electrons, whereas in other ferric compounds the number of unpaired electrons in one iron atom is usually either five or one. The ferric hydroxide precipitated from ferritin by NaOH has the same magnetic characteristic.

The facts known so far are scarcely compatible with the assumption that one has to deal with a stoichiometrically well defined iron salt, or iron complex compound, of apoferritin. Very likely, micelles of colloidal iron hydroxide of composition mainly FeOOH, containing small amounts of ferric phosphate in addition, are interspersed in the open spaces of the very loose crystalline structure of apoferritin. Why a special protein is needed for the storage of the iron can not be explained as yet. It is however obvious that iron is accumulated in the form of ferritin as hemoglobin is broken down, and that ferritin furnishes iron for the formation of fresh hemoglobin. It is remarkable that the iron of ferritin is always in the ferric state, that of hemoglobin however in the ferrous state.

Theory of metachromatic staining. L. Michaelis.

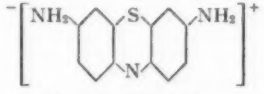
Methods of differential staining, as used in histological technique, may be divided into two classes. The substrate to be stained may be subjected to two (or more) different dyestuffs, either simultaneously or successively, whereby different histological elements absorb selectively the one or the other dye: selective staining. Or, one individual dyestuff may be used to stain various histological elements in different shades of colors: metachromatic staining. Among

those dyestuffs exhibiting the metachromatic effect, toluidine blue and thionine are best known. They stain, for instance, all nuclei, or the cytoplasm of lymphocytes, blue; however the same dyes stain the granula of basophilic leucocytes, or mucus, or "amyloid" pathologically occurring in liver and other organs, purple. This difference in color does not depend on pH within wide limits and should be well distinguished from the pH-effect of indicator dyes. All substrates which stain "metachromatically," instead of "normally," are half-esters of sulfuric acid with high-molecular carbohydrates, either linked to a protein or not, e.g. chondroitin sulfuric acid, mucoproteins, and many vegetable colloids among which agar is best known. Metachromatic dyes, therefore, represent a specific chemical reagent that can be used microscopically in situ. Many more dyes exhibit this effect, if the color analysis is carried out spectrophotometrically. It is a more accidental property of the two dyes mentioned above, to reveal the metachromatic effect directly to the unaided dye. On spectrophotometric observation, methylene blue shows this effect at least to the same extent, though it is not very obvious for the unaided eye.

A comparative study of many dyestuffs has revealed that all those dyestuffs stain metachromatically which, in aqueous solution, have the property of forming polymers of the dyestuff molecules in equilibrium with the monomeric dyestuff molecule. Even in rather dilute solution, such dyestuffs form dimeric molecular aggregates, which can be recognized by the fact that the dimers have an absorption band at a wave length different from that of the monomer. Since the percentage of dimerised dye molecules increases with increasing concentration, the molar absorption coefficient, plotted against wave length, varies with the concentration. Such dye stuffs are said to "disobey Beer's law," according to which the molar absorption coefficient should be independent of concentration.

The absorption curve of the metachromatic color, however, is not the same as that of the dimeric dye, rather is the absorption band still more displaced. There is evidence that the metachromatic color is due to high-polymers of the dye. The difference between normal and metachromatic staining, then, consists in the fact, that the surface of "normally staining" substrates adsorbs a monomolecular layer of the dye, and the surface of metachromatically staining "substrate" absorbs a polymolecular layer. All dyestuff molecules are long, flat, almost two-dimensional molecules. Di- and polymerisation consists in piling up these flat molecules plane to plane. The forces which bring about polymerisation are, in a loose sense, comparable to what we may call exaggerated van der Waal's forces. In every case, the metachromatic color turns to the normal color by increasing the temperature, in a reversible way, due to the fact that thermal motion disrupts the aggregates of the dye molecules.

The correlation of the polychromatic effect of a dyestuff with its chemical structure will be discussed in some other place. The following characteristic example may be mentioned. Thionine contains two amino groups at the ends of the elongated molecule, an electric charge oscillating from the one to the other amino group ("resonance"). It is highly metachromatic. Substituting O for S (oxomine) almost abolishes metachromasy. Eliminating one of the two amino groups (monoaminothiazine) destroys metachromasy entirely.



formula of thionin
(univalent cation, as existing in
neutral or slightly acid solution)

The chemical organization of the cytoplasm. Arnold Lazarow.

The organization within the cell permits the coexistence of otherwise incompatible substances such as phosphatase and certain coenzymes. Although the mechanism is little understood, cell organization is determined, in part, by the localization of enzymes within the cell.

The cytoplasm of the liver cell contains several granular components. These may be separated by differential centrifugation after cell fragmentation. The larger particles are the mitochondria. The smaller particles, which are submicroscopic in size, are of two distinct types —one is particulate glycogen, the other is a lipo-protein complex which can be differentiated from the mitochondria by quantitative chemical analysis. Most of the cytoplasmic lipids are

concentrated in the mitochondria and submicroscopic lipo-protein particulate. Both contain ribose nucleic acid.

These particulates serve as centers of enzymatic localization within the cell. Since both mitochondria and the submicroscopic lipo-protein particulate oxidize succinic acid, they must contain at least three of the respiratory enzymes—succino dehydrogenase, cytochrome C, and cytochrome oxidase. The mitochondria in addition contain glutamic dehydrogenase. Spatial orientation of the components of the respiratory chain, within the particle, may serve to direct metabolic activity.

The manifestations of a reversible structural framework within the cytoplasm, as evidenced by thixotropy, birefringence (at times), and sol-gel transformations may be explained by the existence of a thread-like micelle similar in type to the tobacco mosaic virus. These elongated micelles can produce a rigid structure even though they are spatially separated by 150Å (a distance several times the diameter of an albumen molecule).

Thus although cytoplasm is organized into particulate components (significant for the localization of some of the respiratory enzymes), micellar components (which may give rise to a reversible structural framework), it nevertheless has a continuous aqueous phase.

August 1

Native protein crystallography and diffraction patterns. Dorothy Wrinch.

Any attempt to understand how native proteins operate in living systems must be based on a knowledge of their atomic organization and of the architectural patterns which characterize them. Unfortunately the instability of these molecules precludes all but the most delicate methods. So far the best and perhaps the only techniques that do not destroy the structure we wish to study are those of physics, particularly X-ray diffraction investigations of protein crystals.

While the protein molecule is not known to be biologically active in crystalline form, a full analysis of insulin crystals (for example) would indicate the nature of the surface pattern of the constituent molecular units. It is confirmed by recent crystallographic work (Fankuchen, *Ann. N. Y. Ac. Sci.* **41**: 157; 1941) that the surface pattern of each protein species, as indicated by the interlinking of units in the crystal, is a highly specific and individualistic tapestry of atoms and electrons. Such molecules can crystallize with vastly different complements of foreign molecules (often water) and for their stability depend on this foreign population. Moreover, irrespective of the foreign element, the protein molecule interlinks with its fellows in characteristic ways and maintains intact its own skeletal structure. In striking contrast to many complex organic crystals, the native proteins in general form crystals of high symmetry.

While these studies have yielded important information about molecular weights and some information about shapes and sizes, they have not as yet uncovered the atomic structure of the native protein particle. To obtain any light on this problem from these studies, it is necessary to interpret the intensity data obtained from X-ray observations. This is a matter of the greatest difficulty.

One line of attack is to study the nature of diffraction patterns in general, a problem of absorbing interest to astronomers for more than a hundred years. Diffraction patterns of apertures of various shapes, repeated in various arrangements, are available for study. It is suggested that the study of such patterns as these may enable us to begin to learn the language of diffraction patterns. A more fundamental attack is also suggested, namely the methodological study of the diffraction patterns of distributions of each and every type. An introduction to such studies by the present writer is in course of publication. The aim is to obtain a clear picture as to how structural features of a known distribution manifest themselves in structural features of its diffraction pattern. This correlation of distributions with their diffraction patterns constitutes an assemblage of mathematical facts essential for the adequate exploitation of the experimental data.

The role of adenylpyrophosphatase in alcoholic fermentation of yeast. Otto Meyerhof.

The Naples Station still lives! Ernst Scharrer.

August 8

On the energy source of the nerve action potential. David Nachmansohn.

In earlier theories acetylcholine (ACh) was supposed to be a "synaptic" transmitter, i.e. a substance released at the nerve ending and acting direcly on a second neuron or on the effector cell. According to the new concept the release and the removal of ACh is an *intracellular* process occurring everywhere at the neuronal surface and directly connected with the nerve action potential. The action of ACh may be pictured in the following way: The nerve is surrounded by a polarized membrane. The polarized state of the membrane is due to a selective permeability to K, which is present in different concentrations on either side of the membrane. During the passage of the impulse the resistance of the membrane is decreased and the permeability to all ions increased. Hereby a local depolarization occurs. This change in permeability appears to be produced by the rapid appearance and removel of ACh. The polarized point becomes negative to the adjacent region and flow of current results. This flow of current stimulates the next following point. There again ACh is released and the whole process repeated. The impulse is thus propagated along the axon. At the nerve ending the surface is increased, the resistance therefore decreased. This leads to a greater flow of current which enables the impulse to cross the non-conducting gap. The transmitting agent is always the electric current, the action potential, but the current is generated by ACh. The picture is consistent with the idea of propagated impulses as developed by Keith Lucas and Adrian. It makes unnecessary to assume that the transmission along axon and across synapses differs fundamentally.

If the release and the removal of ACh are responsible for the alterations of the nerve membrane during the transmission of the nerve impulse, chemical reactions must supply the energy for the resynthesis of ACh. The electric organ of *Electrophorous electricus* offers a suitable material for comparing electrical and chemical changes connected with the action potential since both are in the range of possible measurement.

Such measurements were carried out during the last two years (Nachmansohn, Cox, Coates and Machado). The electric energy released per gram and impulse was found to be eight microcalories, the total electric energy about 48 microcalories. The energy released by phosphocreatine breakdown is about 32 microcalories, that by lactic acid formation 15–18 microcalories. Since the energy of lactic acid formation is probably used, as in muscle, to phosphorylate creatine ("Parnas reaction"), these figures are consistent with the conclusion that phosphate bonds may yield the energy for ACh synthesis.

The amounts of ACh which may be split by one gram of electric tissue during one discharge is about 5×10^{-6} millimole. The amount of phosphocreatine actually split per gram and impulse is about 3×10^{-6} millimole. Thus the amounts of ACh and phosphocreatine metabolized seem to be of the same order of magnitude.

One of the facts supporting the new concept is the extremely high concentration everywhere at the neuronal surface indicating a rate of ACh metabolism sufficiently high to parallel the electric changes. In electric tissue the rate may be at least 100,000 times but probably one million times as high as that of respiration. But we have to distinguish between the possible rate and the absolute amounts metabolized. ACh is released and hydrolyzed within a very short period. The recovery requires one to two hours during which the rate of respiration may be increased. If the absolute amounts are compared, a satisfactory picture is obtained.

A whole chain of reactions connected with the nerve potential could be established. Since it is initiated by the release of ACh, it has been called the "acetylcholine cycle."

As a result of these investigations a new enzyme, choline acetylase, could be extracted from brain which in presence of adenosine triphosphate under strictly anaerobic conditions and in cell-free solution synthesizes ACh.

Current, voltage, and resistance characteristics of injured nerves.[1] Abraham M. Shanes.

Recent studies of the less active aspects of bioelectricity have been almost completely restricted to the "resting" or "injury" potentials. However, if the currents these potential differ-

[1] Aided in part by a grant from the American Academy of Arts and Sciences.

ences produce are also considered, information is obtained which is useful for an understanding of the nature of the potentials and for a study of the permeability of the cell membrane and the conductivity of the protoplasm.

Under the conditions of measurement (injured distal end of nerve in Ringer isotonic KCl solution and ca. 2 cm. of nerve between injured and uninjured regions) currents of 0.2–0.4 microampere are obtained when the injured and uninjured areas of frog sciatic nerve are shorted together. The amount of current drawn can be changed by introducing resistance in the external circuit. Thus it is found that the voltage between the two regions of the nerve varies linearly with this current under all experimental conditions which have been examined. On the basis of Thevenin's theorem it can be concluded that the internal e.m.f. as well as the resistance elements of the nerve fibers are constant up to the largest obtainable currents. This is equally true of the leg nerves of spider and blue crabs, where currents up to 5.5 microamperes are produced.

It is therefore possible to define an internal resistance R as the ratio of the resting potential (i.e., the voltage without external current drain) to the current obtained with a short circuit. In frog nerve this is 100,000 ohms or more, while in invertebrate nerve it measures $\frac{1}{5}$–$\frac{1}{10}$ as great. Preliminary experiments with frog nerve demonstrate that anoxia and excess KCl produce a 4–6 per cent decrease in R, presumably because of changes in the axoplasm, the membrane, or both.

A simple extension of these current and potential measurements makes possible the separate determination of the membrane and protoplasm resistances. Under the conditions of measurement the membrane resistance of frog nerve is 5–10 per cent of the total, while in crab nerve it is insignificant compared with the axoplasm. This difference between the two types of nerves is to be expected from their different structure. The membrane resistance disappears when either is allowed to lower the resting potential to the point of irreversibility. The accuracy of the resistance measurements in the case of frog nerve is indicated by the close agreement of R, measured as described above, with the sum of the separately determined resistances, the agreement usually being within three per cent or better.

Oscillographic studies on the giant nerve fiber system in Lumbricus. Theodore H. Bullock.

This system consists of a median and two lateral fibers on the dorsum of the cord, each comprising a chain of compound axons, separated by septa in each segment. The laterals anastomose frequently. The system mediates the rapid, twitch-like response to startle stimuli and the fibers are said to be polarized, the median conducting backwards, the laterals forwards.

Eccles, Granit and Young (1933) were the first to record the activity of these fibers. This work completely confirms theirs. When the cord is stimulated electrically a consistent pattern of two giant action potential spikes may be recorded anywhere along its length. Above the threshold of these two increase in strength has no effect. The pattern of two agrees with the expectation from anatomy. Further evidence that the giant spikes are related to the giant fibers has been obtained by local thermocoagulation and puncture of single giant fibers. The median fiber has the faster, smaller spike. Speed of conduction of this fiber is of the order of 25–50 m./sec., taken as the elapsed time between stimulus and spike over a gross distance measured on the outside of the animal, i.e. these are minimum figures. They depend on the state of contraction, the rate going up as the animal is stretched, but not proportionally; elapsed time also goes up. The slower fiber conducts at one-third to one-half the rate of the faster. Both are going so fast that there is no significant time available to ascribe to synaptic delay: approximately five milliseconds of elapsed time must be divided between, presumably, about a hundred synapses and about 20 cm. of conduction distance. The pattern of response and the speeds of conduction are the same antero-posteriorly and postero-anteriorly. The two spikes in each direction must be carried by the same fibers. Impulses coming in one direction can block those coming in the other. If simultaneous stimuli are delivered to the cord near the two ends of the animal and the responses are picked up near the middle, two spikes, coming from the nearer stimulating electrodes are recorded, instead of four. The giant fibers, including whatever segmental synapses they have, must be unpolarized. Normal mechanical stimuli at the skin result in the same spikes, but only one spike type from any one site of stimulation: the small, fast spike from a stimulus anywhere in the anterior third of the worm, the large, slow spike from the posterior two-thirds. A sharp line separates the two regions, just behind the clitellum.

Whichever spike is elicited can be detected both in front and behind the site of stimulation, i.e. it is conducted in both directions from this point. The fibers are unpolarized but evidently there are afferent cells connected to the median giant only in the anterior third and connections capable of setting off the lateral giants only in the posterior two thirds. This would explain the apparent polarization found by earlier workers.

Evidence of perpetual proximo-distal growth of nerve fibers.[2] Paul Weiss.

G. H. Parker has postulated a proximo-distal shift of substance in nerve fibers. That some such process really occurs is demonstrated by the following experiments. When a mammalian nerve is chronically constricted by a cuff of artery, the axis cylinders proximal to the constriction assume characteristic shapes, ranging from simple "beading" to ballooning, telescoping, and coiling. These changes (noted in over 50,000 fibers) are most marked immediately at the "bottleneck" and grade off proximally. They appear within a week, and are still present eight months later. They do not perceptibly interfere with nerve function.

The observed configurations resemble closely the damming up of a column of viscous substance driven forward against elastic resistance. One is thus led to the following concept: The neuron, as a living cell, is in a state of constant reconstitution. The synthesis of its protoplasm would be confined to the territory near the nucleus (perikaryon). New substance would constantly be added to the nerve processes from their base. The normal fiber caliber permits unimpeded advance of this mass, with central synthesis and peripheral destruction in balance. Any reduction of caliber impedes proximo-distal progress of the column and thus leads to its damming up, coiling, etc.

This concept is supported by two facts. Firstly, the spacing of the beads (4000 measurements) increases in linear proportion to their distance from the constriction, which is precisely the form to be expected from models. Secondly, release of the constriction after several months is followed by a gradual centrifugal spreading of the dammed up substance with straightening and equalisation of caliber of the affected fibers.

Rate of growth and final caliber of a regenerating nerve fiber vary with the rate of supply from the central cell body. Nerve fibers which have grown through a constricted zone remain small and poorly myelinated in the parts lying distal to the constriction (Weiss and Taylor, *Proc. Soc. Exp. Biol. and Med.*, **55**; 1944).

Our experiments suggest that reproduction of the basic neural protoplasm occurs only near the nucleus. If all cells behave in this manner, this would mean that cytoplasmic reproduction does not occur throughout the cytoplasm, but only in the vicinity of the nucleus, a fact which would have far-reaching implications for our concept of growth.

August 15

A toxic substance from protoplasm. L. V. Heilbrunn, D. L. Harris, P. G. LeFevre, W. H. Price, W. L. Wilson, and A. A. Woodward, Jr.

The chemical nature of a toxic substance obtained from protoplasm. D. L. Harris, W. H. Price, and L. V. Heilbrunn.

Recent developments in ultraviolet microscopy. George I. Lavin.

In 1904 Köhler (*Zeit. f. wiss. Mikros.*, **21**: 129) described a quartz microscope in which the objectives were corrected for the 2750 Å line. The reason for employing such an instrument is that nucleic acid, the proteins, and nucleoproteins have characteristic absorption bands in the ultraviolet region of the spectrum, so it is to be expected that unstained tissue photographed under this condition would show structure not apparent with visible light. Since the resolution is directly dependent on the wavelength of light used, photographs taken with ultraviolet light can be greatly enlarged without undue loss in structure.

[2] This research was done under a government contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. It was aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Although work has been done with the original set-up the instrument has been difficult to use owing to the inability of obtaining a satisfactory focus and field. A simplified arrangement has been described (Lavin, *Rev. Sci. Instr.*, **14**: 373; 1943) in which the light source is a resonance mercury vapor lamp (2537 Å), a cobalt sulfate-nickel sulfate filter is used to take out the visible light, and the focussing is carried out with the aid of a willemite screen.

A number of materials such as cancer cells, muscle (Hoagland, Shank and Lavin, *J. Exptl. Med.*, **80**: 9; 1944), nerve cells, marine animal eggs (Harvey and Lavin, *Biol. Bull.*, **86**: 163; 1944) red cells, etc., have been photographed, and the indications are that the instrument will have considerable application since it makes possible the easy study of unstained and in certain cases unfixed tissues.

August 22

Peripheral circulatory changes during shock produced by hemorrhage. Benjamin W. Zweifach.

The reactions of the capillary bed, representing an organic unit directly concerned with the circulatory failure characteristic of shock, have proved a valuable and sensitive index of the state of the animal throughout the syndrome. Various types of hemorrhage were carried out on dogs of which the omentum had been exteriorized to microscopic observation.

Three types of peripheral circulatory collapse could be produced when hemorrhage alone was the initiating factor. The first is represented by acute hemorrhage, with which no essential dysfunction of the capillary bed occurs. The circulatory collapse is the result of the excessive vasoconstriction of the larger blood vessels and a mechanical failure of the blood to reach the tissues.

The second or intermediate type occurs with graded hemorrhage in which the dog is maintained in a hypotensive state for an extended period and in which there appears progressively a physiologic derangement of the peripheral circulation. This latter condition serves to intensify the general oligemia by the pooling of blood in the capillary vessels and in the small venules.

The third or toxic type was obtained when ether was used as the anesthetic agent, instead of pentobarbital as in the previous experiments. The circulatory collapse involved, in addition to a physiologic derangement, an increase of the normal endothelial permeability, and terminated in true capillary stasis and hemoconcentration.

It is interesting to note the correlation between the degree of dysfunction of the capillary bed and the response to transfusion therapy. The acute hemorrhage type of circulatory failure is responsive to almost any type of fluid infusion, even saline. The second type which involves a progressive derangement of the capillary circulation becomes progressively refractory to crystalloidal fluids, then to colloidal blood substitutes (gelatin, albumin), and finally even to plasma or whole blood. The third type, which possesses the most marked abnormality of the capillary circulation, is least responsive to fluid therapy, over 85 per cent of these animals being refractory to transfusion procedures.

Recent experience with hemoglobin-saline solutions. William R. Amberson, Joye J. Jennings and C. Martin Rhode.

Efforts have been made to prepare and preserve human hemoglobin in a form suitable for intravenous injection into clinical cases. It has not been possible to prepare a dry lyophile product suitable for therapeutic use when redissolved. Such a solution always contains much methemoglobin and some insoluble denatured protein. It is, however, possible to preserve hemoglobin without desiccation or refrigeration by placing sterile solutions in glass vessels which are then rendered oxygen free by evacuation, and sealed. In such solutions methemoglobin formation and denaturation do not occur. In the course of the preparation the stromata must be removed by ether or toluene extraction, and the potassium by dialysis. A pyrogen-free product has been prepared.

Hemoglobin solutions of this type have two possible therapeutic uses. In five human cases of anemia stimulation of hematopoiesis has been observed, after the infusion of 200 to 500 cc. of hemoglobin-saline daily over a number of days. A similar effect has long been known in

animals but no human observations have previously been reported. A second use of the solution may be the treatment of certain types of shock. In a single case of shock, caused by massive hemorrhage after childbirth, hemoglobin-saline solutions raised blood pressure and caused return of consciousness after plasma and whole blood infusions had failed to terminate the condition. The patient died with anuria on the eighth day. The case affords hope that such solutions may prove of value in the treatment of shock after hemorrhage, since they are able to transport oxygen as well as to maintain the colloidal osmotic pressure of the blood. Lost blood volume is thereby restored, and the blood pressure returned to normal.

Gelatin as a plasma substitute, with special reference to pseudo-agglutination. Richard G. Abell and William M. Parkins.

That gelatin possesses many of the properties desirable in a plasma substitute has recently been demonstrated by Parkins, Koop, Riegel, Vars and Lockwood (1943). The present investigations on pseudo-agglutination are a part of a larger program being carried out at the University of Pennsylvania by Dr. Parkins and others in which various properties of gelatin in reference to its use as a plasma substitute are being further investigated. The results to be described in this abstract have previously been mentiond briefly in the paper by Parkins et al. referred to above.

It is well known that the addition of gelatin to erythrocytes in vitro causes these cells to adhere in clumps. This phenomenon, known as pseudo-agglutination, has been reported to follow infusions of gelatin by Hanzlik and Karner (1920), Amberson (1937), Stein, Grodins and Dutton (1943) and Grodins (1943). If such clumps are formed within the blood vessels, it is important to know whether they interfere with the blood flow.

Six rabbits and five dogs were infused with six per cent solutions of biological gelatin (courtesy of Dr. D. Tourtellotte, Charles B. Knox Gelatin Co., and Kind and Knox Gelatin Co.) in 0.85 per cent saline, and the effect upon the blood vessels and blood flow observed directly with the microscope in the living animal. In the case of the dogs, the vessels studied were inclosed within intestinal-mesenteric chambers, modified to fit the dog from the original type described by Zintel (1936). Following acute hemorrhage to the point of reducing the blood pressure to 30 mm. Hg, the blood in many of the capillaries and venules became stationary; in the arterioles the flow became sluggish. Replacement of the blood lost by hemorrhage with an equal volume of gelatin caused the blood flow to return to its control rate.

In the normal rapid flow in the dog's mesentery no clumps could be detected following gelatin infusion. Only when the rate of flow was decreased and stagnation induced (by further hemorrhage) could such clumps be seen.

In order to secure further evidence on the manner in which gelatin causes pseudo-agglutination of erythrocytes, transparent moat chambers (Abell, 1932) were inserted in rabbits' ears. After these chambers became vascularized, six per cent solutions of gelatin were injected intravenously (15 cc./kg.) and the effect studied with the microscope.

No pseudo-agglutination could be observed until the rate of flow was reduced by squeezing the main artery of the ear, which supplied the vessels in the chambers. When this was done, it could be seen that erythrocytes that came together side by side adhered to each other to form groups of cells in rouleaux. In control experiments this occurred normally, in the absence of gelatin. Following gelatin infusions, however, the individual rouleaux groups adhered to each other to form larger clumps, made up of several rouleaux groups.

Following gelatin injections, the erythrocytes were seen to pass from the arterioles into the capillaries as separate cells. They did not adhere to each other to form pseudo-agglutinated clumps to any appreciable extent, until they reached the venules. Such clumps did not interfere with the blood flow. When a clump approached a vessel smaller than it was, it separated into its constituent cells, and hence did not block the vessel. The clumps floated in the plasma, which carried them along, and which was always between them and the walls of the vessels. There was no evidence of increase in viscosity of the blood.

Thus, although pseudo-agglutination of erythrocytes does occur following intravenous injections of gelatin, such pseudo-agglutinated cells do not block the vessels or interfere with the blood flow. Consequently, from the standpoint of flow, pseudo-agglutination does not contraindicate the use of gelatin as a plasma substitute.

August 24

Experimental studies on the cytology of allium. C. A. Berger.

The cytological effects of a number of chemical agents were studied in the root tips of *Allium cepa* and compared with the well known effects of colchicine. Acenaphthene, veratrine, sulfanilamide, chloral hydrate and benzene all induced polyploidy and produced cytological effects similar to those of colchicine.

The primary effects of all these substances are the prevention of the formation of an effective spindle and a delay in the division of the spindle attachment regions of the chromosomes. As a result of these primary effects the chromosomes are held at metaphase and become shorter and thicker than normal metaphase chromosomes. After a longer or shorter delay the spindle attachment regions divide, but in the absence of an effective spindle no anaphase movement takes place and the whole group of chromosomes undergoes a revision process giving rise to a tetraploid resting nucleus. After a sufficient period of recovery these cells undergo mitosis as polyploid cells.

Root tips grown in an atmosphere lacking oxygen were found to show similar cytological effects and to produce tetraploid cells. The tentative conclusion is advanced that these effects are not specific to any of the chemicals in question but are general effects common to all the substances in question and interfering with some fundamental metabolic process concerned with the formation of the spindle and the division of the spindle attachment region.

Naphthalene-acetic acid was also used. This substance does not affect the meristem but induces polyploid divisions in the older, differentiated regions of the root. Naphthalene-acetic acid differs from the other substances in that it does not affect cells in division, but causes a double reproduction of the chromosomes in the resting nuclei. At metaphase tetrachromosomes are found. These are four chromosomes held together at a common undivided spindle attachment region. After a slight delay at metaphase two successive divisions of the spindle attachment regions occur. Naphthalene-acetic acid does not inferfere with the formation of the spindle and anaphase separation occurs, resulting in two tetraploid cells.

Studies on the chemical basis of fever. Valy Menkin, M.D.[3]

Fever is usually associated with some form of cell injury. Inflammation is the complex vascular, lymphatic, and tissue response in vertebrates to the presence of an irritant and as such, it represents a manifestation of severe cellular injury. The pattern of injury in inflammation has been shown in earlier studies to be referable to a thermolabile, non-diffusible substance located in the euglobulin fraction of exudates. (*Arch. Path.*, 1943; **36**: 269.) This substance, termed necrosin, appears to be either a proteolytic enzyme or else to have proteolytic activity associated with it. Necrosin, in the form of the toxic euglobulin of exudates, is pyrogenic to both dogs and rabbits. (*Proc. Soc. Exp. Biol. and Med.*, 1943; **54**: 184; *Fed. Proc.*, 1944; **3**: No. 1.) Its formation at the site of injury and its absorption into the circulation offers a reasonable explanation for the basic mechanism of fever accompanying numerous inflammatory processes.

Recent studies indicate that this toxic euglobulin contains a component in turn insoluble in the presence of NaCl or $SO_4^=$. This component is essentially the fever-inducing factor or at least it is associated with that fraction. It can be dissociated from necrosin by treating the exudate with ammonium sulphate at one-third saturation. The precipitate formed is treated with distilled water prior to dialysis of the $SO_4^=$ ions. A true euglobulin enters into the aqueous phase containing the $SO_4^=$ ions. This is necrosin in a further state of purification. It is toxic to mice and is capable of inducing a severe cutaneous inflammation; but it is non-pyrogenic. The pyrogenic factor seems primarily associated with the precipitate which has failed to dissolve in the aqueous phase containing the $SO_4^=$ ions. This highly fever-inducing substance, readily dried by freezing, is termed "*pyrexin.*" Its presence offers a satisfactory explanation for the basic mechanism of fever with inflammation. It is thermostable. Boiling fails to inactivate pyrexin. Ashing destroys it. Incubation of the non-pyrogenic purified necrosin favors the formation of pyrexin. This suggests that pyrexin may be an end product of proteolysis associated with enzymatic activity in the necrosin fraction. It is absent in non-hemolyzed serum, but it is present

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to some extent in hemolyzed serum and in serum from an animal with a concomitant acute inflammation. It is absent in the pseudoglobulin and albumin fractions of exudates. Pyrexin is excreted, at least in part, in urine. The N and P contents of pyrexin are about ten per cent and one per cent respectively. The material is Biuret negative but Ninhydrin positive, except in the fraction recovered from urine which is usually also Ninhydrin negative. It is Molisch negative. It is insoluble in ether and 95 per cent alcohol, but apparently soluble in relatively weak alkali. The possibility of a peptide attached to a nucleic acid derivative is not precluded by the available data. The exact chemical nature of pyrexin is, however, unknown, and will therefore require further studies. Evidence with barbiturates and antipyretics indicates that the possible mode of action of pyrexin is on the fever centers in the hypothalamic region.

In vitro fertilization and cleavage of human ovarian eggs. John Rock and Miriam F. Menkin.

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Phosphoprotein phosphatase, a new enzyme from the frog egg. Daniel L. Harris.[4]

Immediately following homogenization of the ovarian eggs of the leopard frog, *Rana pipiens*, in 0.1 M sodium citrate, there is a rapid liberation of inorganic phosphate from some ester within the eggs. The reaction takes place over a broad pH range in neutral or acid solutions, but there seems to be relatively little hydrolysis in an alkaline medium. There is a pronounced optimum at or near pH 5.0. At pH 5.0 the inorganic phosphate rises from about 23 mg. per cent to 250 mg. per cent in 5 minutes. The speed of the reaction as well as the pH optimum indicates that the hydrolysis is due to an enzyme rather than to the acid conditions. Furthermore, the activity is destroyed by heating.

An analysis of the changes in distribution of phosphate in the brei following "autolysis" as compared with the control in which the reaction was prevented by the addition of trichloracetic acid showed the following: There was no significant change in phospholipid, an increase instead of the expected decrease in organic acid-soluble esters, but a profound decrease in phosphate bound to protein. This decrease in protein phosphate was sufficient to account for the increase in inorganic phosphate as well as the increase in organic acid-soluble phosphate esters. The latter esters have not been identified as yet, but they are known to be resistant compounds, withstanding 1 N HCl for three hours at 100 ° C.

Nucleoproteins and the phosphoproteins of the yolk platelets are the main proteins containing phosphate in the frog egg. To distinguish between these two possible substrates, tests were made using nucleic acid and casein, a typical phosphoprotein, as substrates. There was a rapid liberation of phosphate from casein, but little or none from nucleic acid. Indeed, nucleic acid appeared to inhibit the action of the enzyme on the natural substrate. Vitellin, isolated from the yolk of hen's eggs, was attacked as were yolk platelets which were denatured by heat and added in excess. Sodium β-glycerophosphate and disodium phenylphosphate were hydrolyzed but slowly if at all. The enzyme is, therefore, regarded as a phosphoprotein phosphatase, and the natural substrate within the frog egg is yolk. In the developing embryo the enzyme is thought to liberate inorganic phosphate as needed for carbohydrate metabolism.

The enzyme is bound, in part at least, to the yolk platelets. Lack of contact between the enzyme and substrate can not, therefore, explain the fact that the hydrolytic action of the enzyme is held in check in the ovarian eggs which remain essentially unchanged throughout many months.

Behavior and tube building habits of Polydora ligni. Edith Mortensen and Paul S. Galtsoff.

Polychaete worms of the genus Polydora live on mud bottoms where they cause profound changes by gathering and depositing huge quantities of mud, often covering and smothering other

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inhabitants with a thick layer of material formed of their loosely constructed mud tubes and excreta. The worms also penetrate between the mantle and the shell of oysters where so-called mud blisters are formed. Thus they sometimes change from a free living to a commensal existence.

In tube building the mud is gathered in a deep ciliated groove extending the length of the inner margin of each of a pair of tentacular cirri as they lash about and secrete a sticky mucus. As the worm turns on its long axis, the mucus-covered mud is passed to the basal end of the tentacles and then is dropped, forming a ring about the anterior end of the body. The mucus is sufficiently adhesive to cause the particles of mud to stick together readily and immediately. There is no systematic placing of the mud in a rotary fashion at the edge of the tube, nor is the material packed together in any way.

That tubes are essential for the survival of *Polydora ligni* was demonstrated in experiments in which two groups of 20 animals were kept under identical conditions with the exception that one group was kept in glass tubing whereas the other was placed free in sea water. On the 23rd day of the experiment, all of the 20 worms left free had perished while 16 of the 20 kept in tubes were still alive.

Rejection of material unsuitable for tube building or food is accomplished by the reversal of ciliary motion along the tentacular groove. When an animal is given substances such as corn starch, Chinese ink, finely ground glass, and sand grains of various sizes, the number of reversals recorded per unit of time is greater than when the same animal is given mud from its natural environment. Likewise the number of reversals occurring when mud soaked in a M/40 KCL solution is used is greater than with mud alone. A 0.01 per cent lactic acid solution added to corn starch gives more reversals than corn starch alone. Thus physical and chemical factors control the reversal of ciliary motion.

The funnel shaped pygidial structure at the posterior end of the body is not a sucker as has been suggested by previous authors but probably a plunger for clearing the tube of excreta.

The conditions which stimulate the entrance of the worms into oysters and cause the free living animal to assume a commensal habit remain unknown. Apparently the worms are not attracted by the oysters and, as laboratory experiments show, may remain in close association with them without penerating their shells. Infestation of oysters by Polydora may be a purely accidental phenomenon.

The click mechanism in elaterid beetles. J. B. Buck.

Several thousand tests on four species of elaterids showed that in click-jumping from an initial dorsum-down position the beetles come to rest on their feet about twice as often as on their dorsal surfaces.

In testing the possibility that this is achieved by controlling the number of aerial loops or twists so that an upright landing results, it was found that: (1) Varying the ratio of height jumped to distance fallen caused no consistent change in the percentage of "successful" (upright) jumps. (2) A tabulation of the direction in which the beetles were facing after jumping indicated that a position in the original plane of the longitudinal axis is favored, and that among the successful jumps those which end with the beetle facing the same direction as originally are somewhat more common than expected. Such a position could only result from a jump including $n + \frac{1}{2}$ twists (rotation on the longitudinal axis) and $n + \frac{1}{2}$ loops (rotation on the transverse axis).

In testing the alternative possibility that the excess of successful jumps is due to body shape or weight distribution, beetles were shaken in a box and dropped or thrown on to a level surface. Beetles dropped in such random fashion land upright about as frequently as after normal jumps, and moreover it makes no difference whether the beetle is dead or alive. Success is enhanced—sometimes to 85 or 90 per cent—by jumping or dropping the beetles on inclined planes, indicating that anything which increases the probability of rolling or bouncing increases their chance of reaching the upright position.

The heights reached in jumping are distributed normally, and the proportion of successful jumps is the same in each height class, so that if success depends on completing a particular number of aerial loops or twists, that number is a constant, independent of the height reached.

Changes in temperature do not affect significantly the proportion of successful jumps.

The evidence summarized indicates that although there may be some path-selection in the mechanism of the jump, the major factor in the preponderance of successful jumps is a predisposition toward the attainment of the upright position, probably due to the external shape of the body.

Metamorphosis in the larva of the Tunicate, Styela partita. L. M. Bertholf and S. O. Mast.

The organisms used in this investigation were kept continuously at 20–21° C.

The average length of larval life (i.e. the time between hatching and the retraction of the tail) varies with the season. It increased from approximately five hours late in June to a maximum of 80 hours early in August and then decreased somewhat. There is great individual variation. It ranged from less than one hour to more than eight days during the course of the summer.

In sea-water in which a few hundred larvae per cc. had previously metamorphosed, the length of larval life is much shorter than it is in normal sea-water. As the number of larvae which had metamorphosed in a given quantity of sea-water increases, the rate of metamorphosis in fresh larvae in this sea-water increases to a maximum and then decreases to zero, i.e. the sea-water becomes so toxic that it kills the larvae before the tail is retracted.

Cupric chloride (2×10^{-6} M.) and Janus green (one part in 250,000 of sea-water), each acting for 3 minutes, greatly accelerate metamorphosis. Neutral red, extract of the muscle of a rabbit killed by x-rays, and concentrated sea-water accelerate it somewhat. Increases in hydrogen-ion concentration from pH 8.05 to pH 7.47, dilution of sea-water up to 40 per cent, crowding of the larvae (1000 per cc. of sea-water) have no measurable effect.

We postulate the following hypothesis of metamorphosis in this animal: It is known that the larva consists of organized adult tissue and organized larval tissue. The larval tissue probably produces a substance which in low concentration augments metabolism and in high concentrations retards it. This substance, then, would be more concentrated in the larval tissue, where it is produced, than in the adult tissue, into which it diffuses. It therefore, at a certain concentration, would retard metabolism in the former and augment it in the latter to such an extent that metamorphosis is initiated.

This substance doubtless diffuses out of the larval tissue into the surrounding medium. Sea-water in which metamorphosis has occurred, therefore, contains some of this substance, which diffuses into fresh larvae put into it, and consequently increases the substance in the larval tissue and thus accelerates metamorphosis. Cupric chloride, Janus green, and other compounds which accelerate metamorphosis probably merely increase the retarding effect of the substance produced by the larval tissue.